THE ROLE OF CYCLOOXYGENASE-2 AND PROSTAGLANDIN E_2 IN THE PATHOGENESIS OF PULMONARY FIBROSIS

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Abstract

Although the pathogenesis of pulmonary fibrosis is incompletely understood, an accepted underlying molecular mechanism is the dysregulation of soluble mediators which regulate fibroblast function in the lung. One such mediator is prostaglandin E_2 (PGE_2), which is a potent inhibitor of fibroblast proliferation, collagen production, chemotaxis and myofibroblast differentiation. Levels of PGE_2 have been shown to be decreased in bronchoalveolar lavage fluid (BALF) and lung fibroblasts from patients with pulmonary fibrosis. In lung fibroblasts this has been shown to be due to limited expression of the rate limiting enzyme in its biosynthesis, cyclooxygenase-2 (COX-2). However, there is currently no direct proof that limited expression of COX-2 and PGE_2 contribute to the pathogenesis of pulmonary fibrosis. This thesis has used pharmacological and genetic inhibition of COX-2 to address the hypothesis that limited expression of COX-2 and PGE_2 potentiates bleomycin-induced pulmonary fibrosis. Through the use of the highly selective COX-2 inhibitor NS398, this thesis demonstrates that bleomycin-induced PGE_2 production in the lung is COX-2 mediated for at least 14 days following injury. However, administration of NS398 had no effect on the development of bleomycin-induced lung fibrosis in wild type (WT) mice. Unexpectedly, COX-$2^{-/-}$ mice showed a compensatory upregulation in PGE_2 biosynthesis following bleomycin injury compared with both WT and COX-$2^{+/-}$ animals, which is evident in macrophage/monocytes but not fibroblasts derived from these mice. Lung homogenates showed increased expression of COX-1 in COX-$2^{-/-}$ mice compared with WT controls suggesting the compensatory synthesis is via increased expression of COX-1. COX-$2^{-/-}$ mice show an enhanced and persistent inflammatory response to bleomycin, however the fibrotic response to injury was unaltered compared with WT animals. In contrast, COX-$2^{+/-}$ mice showed reduced expression of COX-2 and subsequently limited induction of PGE_2 following bleomycin injury. This resulted in an enhanced fibrotic response at day 28 with increased total lung collagen content compared with both WT and COX-$2^{-/-}$ mice (WT, 2.6 ±0.22; COX-$2^{+/-}$, 3.77 ±0.12; COX-$2^{-/-}$, 2.51 ±0.26 mg collagen/lung, $p<0.001$).

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List of Abbreviations

A	Absorbance
AA	Arachidonic acid
AEBSF	4-(2-Aminoethyl)benzenesulfonylfluoride . HCl
AECs	Alveolar epithelial cells
AIP	Acute interstitial pneumonitis
ANOVA	Analysis of variance
ARDS	Acute respiratory distress syndrome
α-SMA	Alpha smooth muscle actin
ATS	American Thoracic Society
b	Basepair
B196	Littermate wild type mice
B197	Strain-matched wild type mice
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
bFGF	Basic fibroblast growth factor
BLA	Bicinchoronic acid
Blm	Bleomycin
BSA	Bovine serum albumin
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
cDNA	Cloned DNA
CFA	Cryptogenic fibrosing alveolitis
CMV	Cytomegal ovirus
CO_2	Carbon dioxide
Conc.	Concentration
COX	Cyclooxygenase
$COX\text{-}2^{+/+}$	Wild type mice
$COX\text{-}2^{+/-}$	Mice heterozygous for COX-2
$COX\text{-}2^{-/-}$	COX-2 gene deleted mice
$COX\text{-}2^{ex1/ex1}$	$COX\text{-}2^{-/-}$ mice generated via disruption of exon 1
$COX\text{-}2^{ex8/ex8}$	$COX\text{-}2^{-/-}$ mice generated via disruption of exon 8
cP450	Cytochrome P450

cPGES	Cytosolic prostaglandin E synthase
$cPLA_2$	Cytosolic phospholipase A_2
CR	Carbonyl reductase
CRE	cAMP response element
CTGF	Connective tissue growth factor
ddH20	Double deionised water
DIP	Desquamative interstitial pneumonia
DMEM	Dulbelcco's modified Eagles medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
DSS	Dextran sodium sulphate
EBV	Epstein Barr virus
ECL	Enhanced chemilumnescent
ECM	Extracellular matrix
EIA	Enzyme immunoassay
ELISA	Enzyme linked immunosorbent assay
EP	PGE_2 receptor
ERK	Extracellular signalling related kinase
ERS	European Respiratory Society
ET-1	Endothelin-1
FBS	Foetal bovine serum
g	Gram
GC	Gas chromatography
GMCSF	Granulocyte-macrophage stimulating factor
H_2O	Water
HCl	Hydrogen chloride
HETE	Hydroxyeicosatetraenoic acid
HFL-1	Human foetal lung fibroblast
HUVEC	Human umbilical vein endothelial cell
HRP	Horseradish peroxidase
HPLC	High performance liquid chromatography
IFN-γ	Interferon gamma
IIP	Idiopathic interstitial pneumonia
IL	Interleukin

I.P	Intraperitoneal
IPF	Idiopathic pulmonary fibrosis
I.T	Intratracheal
Kb	Kilobase pair
KDa	Kilodalton
L	Litre
LIP	Lymphoid interstitial pneumonia
LO	Lipoxygenase
LPS	Lipopolysaccharide
LT	Leukotriene
LX	Lipoxin
MAPK	Mitogen-activating protein kinase
MMP	Matrix metalloproteinase
mRNA	Messager ribonucleic acid
mPGES	Membrane-bound prostaglandin E synthase
MS	Mass spectrometry
MWM	Molecular weight marker
NaOH	Sodium hydroxide
NBD-Cl	7-chloro-4-nitrobenzo-oxaol, 3-diazole
nm	Nanometre
nM	Nanomolar
NS398	N-[-2-cyclohexyloxy]-4-nitrophenyl methanesulphonamide
NSAIDs	Nonsteroidal anti-inflammatory drugs
NSB	Non-specific binding
NSIP	Non-specific interstitial pneumonia
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PF	Pulmonary fibrosis
PG	Prostaglandin
PGDH	15-hydroxy-prostaglandin dehydrogenase
PGE_2	Prostaglandin E_2
PGES	Prostaglandin E synthase

PGHS	Prostaglandin H synthase
PMN	Polymorphonuclear leukocyte
PVDF	Polyvinylidene difluoride
RBILD	Respiratory bronchiolitis and interstitial lung disease
RER	Rough endoplasmic reticulum
RT	Room temperature
Sal	Saline
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
$sPLA_2$	Secreted phospholipase A_2
T_0	Time zero
TA	Total activity
TBE	Tris. borate. EDTA
TBS	Tris. buffered saline
TGF-β	Transforming growth factor -beta
Th	T-helper
TIMP	Tissue inhibitor of metalloproteinase
TNF-α	Tissue necrosis factor
TPA	Tetradecanoyl phorbolacetate
TXA2	Thromboxane A_2
UCL	University College London
UIP	Usual interstitial pneumonia
V_2O_5	Vanadium pentoxide
v/v	Volume per volume
WT	Wild type mice
w/v	Weight per volume
w/w	Weight per weight
x g	Gravitational field

CHAPTER ONE:

INTRODUCTION

1.1 Pulmonary fibrosis

The diagnosis of pulmonary fibrosis (PF) spans a range of interstitial lung diseases of both known and unknown aetiology. It is a chronic and progressive process leading to severe respiratory insufficiency and ultimately death of those affected. Treatment is inadequate and has not altered significantly in the last fifty years. Pulmonary fibrosis is characterised by inflammation, uncontrolled fibroblast proliferation and excessive deposition of extracellular matrix proteins, including collagens, in the lung. However, the mechanisms which drive this process are not fully elucidated.

This chapter will discuss the prevalence, aetiology, clinical features and pathogenesis of pulmonary fibrosis, specifically focusing on idiopathic pulmonary fibrosis (IPF, synonymously known as cryptogenic fibrosis alveolitis, CFA), before examining some of the features common to the fibrotic lung diseases, namely collagen deposition and inflammation in the lung. Following this, specific mediators known to play a role in the pathogenic process of pulmonary fibrosis will be discussed, focusing specifically on prostaglandin E_2 (PGE_2) and its biosynthesis via the cyclooxygenase (COX) pathway.

1.1.1 Incidence of pulmonary fibrosis

An American study reported the incidence of pulmonary fibrosis at 80.9 per 100,000 in males and 67.2 per 100,000 in females (Coultas *et al.,* 1994), and it is thought that over 3000 people die each year from pulmonary fibrosis in the UK alone, with IPF accounting for 1000 of these deaths, and these figures appear to be increasing (Hubbard *et al.,* 1996). IPF occurs more often amongst men than women and in both sexes there is a clear relationship between the development of pulmonary fibrosis and age, where two thirds of patients are over 60 years old (Coultas *et al.,* 1994).

1.1.2 Aetiology of pulmonary fibrosis

The majority of cases of pulmonary fibrosis are of unknown aetiology, for example in IPF, as an end point in eosinophilic granuloma, sarcoidosis, and some diseases affecting multiple-organ systems such as rheumatoid arthritis and systemic sclerosis there is no identifiable cause for the progression to lung fibrosis. However, there are a number of known causes which are diverse. For example, acute lung injury from an identifiable cause, as seen in adult respiratory distress syndrome (ARDS) may progress to pulmonary fibrosis; therapeutic drugs and treatments such as bleomycin or amiodarone;

exposure to radiation during radiotherapy can also cause pulmonary fibrosis; exposure to both organic and inorganic particles such as avian antigens in pigeon fanciers lung; inorganic dusts such as silica or asbestos, and occupational exposure to metal or wood dusts have been shown to increase the risk of developing the disease (Hubbard *et al.,* 1996). A case-control study has also found cigarette smoking to be associated with a 1.6-2.3 fold excess risk of developing the disease (Baumgartner *et al.,* 1997). Infective agents such as viruses and bacteria have also been linked to an increased risk of developing pulmonary fibrosis. Epstein-Barr Virus (EBV), cytomegalo- virus (CMV), tuberculosis, influenza, hepatitis C, mycoplasma and legionella to name but a few, have been proposed to play a role (Egan *et al.,* 1997). Recently, Tang and colleagues examined lung specimens from patients with IPF for the presence of herpesviruses and showed that one or more of four herpesviruses; EBV, CMV, human herpesvirus-7 (HHV-7) or HHV-8, were found in 97% of the specimens from patients with IPF, compared with only 36% of control specimens (Tang *et al.,* 2003).

The vast majority of IPF cases appear to be sporadic and there is no data on the heritability of IPF in the general population, however, genetic predisposition appears to play a role in the development of pulmonary fibrosis. Although rare, familial IPF represents about 3% of all IPF cases (Marshall *et. al.,* 2000a), and pulmonary fibrosis is also associated with pleiotrop ic genetic disorders such as Hermansky-Pudlak syndrome which includes an IPF-like PF among other additional features that are not shared with sporadic IPF patients (Crystal *et al.,* 2002). Furthermore, not all exposures to causal agents result in disease further suggesting a genetic component of the process, for example, only a small group of patients receiving similar doses of bleomycin or amiodarone subsequently go on to develop pulmonary fibrosis (Tisdale *et al.,* 1995), and variable susceptibility is evident amongst workers exposed occupationally to similar concentrations of fibrogenic dusts. This is further supported *in vivo* where inbred strains of mice differ in their susceptibility to fibrogenic agents; C57Bl/6 are fibrosis prone, where as Balb/c are fibrosis resistant after radiation (Ward *et al.,* 1989) or over-expression of TGF-β1 (Kolb *et al.,* 2002). To date, published studies on candidate genes have focused on those involved in host defence and inflammation, particularly polymorphisms that may regulate the functions of upstream cytokines, especially IL-1β and TNF-α (Whyte *et al.,* 2000, see **section 1.3.1**). Most recently, Xaubet and colleagues examined the TGF-β_1 gene, the most pro-fibrotic cytokine known to date (see **section 1.4.5**) and although they found no association to susceptibility to IPF,

polymorphisms in the TGF-β_1 gene were associated with a more rapid deterioration in lung function (Xaubet *et al.*, 2003). Together, these data indicate a potential genetic predisposition which together with an appropriate trigger, may initiate the development of pulmonary fibrosis. Furthermore, the clinical heterogeneity of pulmonary fibrosis in terms of systemic involvement, rate of progression and overall prognosis may also be influenced by genetic factors which, if not causal, may play an important role in development of and/or progression of disease.

1.1.3 Clinical features of pulmonary fibrosis

Onset of symptoms is usually gradual and patients often present late, where symptoms such as dyspnea or cough have often been present for more than 6 months prior to presentation (Turner-Warwick *et al.*, 1980; Johkoh *et al.*, 1999). Diagnosis is confirmed using appearance on chest radiographs and imaging studies (HRCT), results of pulmonary function tests and BALF analysis, and surgical lung biopsy where histopathological features lead to a specific diagnosis. IPF specifically remains a diagnosis of exclusion as clinical features are non-specific and mimic those of a number of other pulmonary diseases. Collagen vascular disease, occupational or environmental exposure, or use of medications associated with pulmonary fibrosis must all first be ruled out as causes before the diagnosis of IPF.

The usual clinical course is gradual deterioration and any successful treatment of pulmonary fibrosis is hampered by its typically late presentation. Current treatment is inadequate despite aggressive therapeutic attempts. Corticosteroids have been used since the 1950's, mainly due to the lack of any effective alternative, however only limited improvement has been reported, with or without the additional use of immunosuppressants (Gay *et al.*, 1998; Selman *et al.*, 1998; Douglas *et al.*, 1998 and Mapel *et al.*, 1996) and this is accompanied by a high incidence of adverse side effects. Despite the medical significance of fibrosis, both in the lung, and other tissues, there are currently no treatments which specifically target the fibrotic process itself.

Prognosis is poor with five-year survival ranging from 30 to 50% (Bjoraker *et al.*, 1998), and average survival for this disease varies between 2.5 and 3.5 years following diagnosis, with or without corticosteroid therapy (Hubbard *et al.*, 1998; King *et al.*, 2001). Specific pathologies on lung biopsy may provide increased survival rates. Recently, it has been reported that the cellular histology seen in non specific interstitial

pneumonia (NSIP, see **section 1.2**) provides a clear survival advantage compared with usual interstitial pneumonia (UIP, Daniil *et al.,* 1999). However, it is clear that together these data suggest that pulmonary fibrosis represents a significant clinical problem with little, if any effective treatment and a poor prognosis for affected individuals.

1.2 Pathogenesis of pulmonary fibrosis

Pulmonary fibrosis has been described as aberrant wound healing in the lung. Physiologically, an acute injury to the lung triggers a programme of repair comparable to that extensively described in skin wound healing (Clark, 1996). An initial inflammatory response leads to matrix production and proliferation of mesenchymal cells needed in the normal repair process. This is subsequently followed by degradation of the injured tissue and apoptosis of repair cells, both mesenchymal and inflammatory, usually leading to a return to normal function. This process is coordinated by the highly regulated expression of soluble mediators which are released by both inflammatory cells and resident lung cells.

However, in pathological conditions, this normal process is disrupted and, like other organs, the lung has a limited capacity for repair after significant injury and cell death. Rather than 'wound-healing' resulting in normal structure and function, the lung has a tendency to replace lost or severely damaged tissue with fibrosis or scar tissue (Kuhn, 1991). In fibrotic tissue, following injury, damage occurs to the alveolar surface of the lung leading to a loss of alveolar integrity, loss of type I epithelial and endothelial cells, damage to the basement membrane and proliferation of type II cells. This is accompanied by inflammatory cell accumulation and fibroblast hyperplasia leading to enhanced deposition of extracellular matrix and ultimately irreversible fibrosis of the lung parenchyma and compromised gas exchange. The process is potentiated by the dysregulation of pro-fibrotic mediators such as TGF-β (see **sections 1.4.4; 1.4.5**) by the altered stromal cell population, activated epithelium and inflammatory cells. Pathogenesis can be affected by severity and extent of injury, aetiological agent and type of inflammatory response (reviewed by Gross & Hunninghake, 2001). However, the specific mechanisms which drive this process are not fully elucidated.

IPF is part of a larger group of pathological diseases of unknown aetiology known as the idiopathic interstitial pneumonias (IIPs). The recent ATS/ERS consensus statement classified the IIPs into seven distinct entities: IPF, non-specific interstitial pneumonia (NSIP), cryptogenic organising pneumonia (COP), acute interstitial pneumonia (AIP), respiratory bronchiolotis-associated interstitial lung disease (RB-ILD), desquamative interstitial pneumonia (DIP), and lymphoid interstitial pneumonia (LIP). IPF is the most common of the IIPs and has a histological pattern of usual interstitial pneumonia (UIP) on lung biopsy (King *et al.,* 2001; International consensus statement; ATS & ERS, 2000). UIP is characterised by a heterogeneous appearance with alternating areas of normal lung and chronic fibrosing interstitial pneumonia. Lung architecture is disrupted, with areas of chronic scarring with honeycomb change or marked smooth muscle metaplasia/hyperplasia, frequent subpleural thickening and areas of fibroblastic foci. Interstitial inflammation is usually mild to moderate, patchy and consists of an alveolar septal infiltrate of lymphocytes and plasma cells, associated with hyperplasia of Type II pneumocytes. Again, levels of fibrogenic cytokines such as TGF-β_1, TNF-α, bFGF and PDGF are increased. (International consensus statement; ATS & ERS, 2000).

From what is presently known about the pathogenesis of pulmonary fibrosis, it is clear that two processes appear to be central in its development and progression; namely the initial inflammatory response to injury, followed by the deposition of extracellular matrix proteins, particularly collagens in the lung. The following two sections will discuss in greater detail the current thinking on the role of inflammation in pulmonary fibrosis and collagen deposition in the lung.

1.3 Inflammation in pulmonary fibrosis

Considerable research has focused on investigating how the inflammatory response in the lung is triggered and which mediators are crucial to the perpetuation of the process. It is widely accepted that rather than resolution of the normal inflammatory response following injury, in pulmonary fibrosis the dysregulation of the early inflammatory response results in progression to chronic and persistent inflammation. This, together with fibroblast proliferation and differentiation into a myofibroblast phenotype, drives the late-stage fibrotic process in the lung leading to severe loss of function. This dogma is supported by a wide range of evidence in both human disease and animal models, however, recent reports have questioned the traditional view of inflammation driving

the fibrotic process. This section will firstly describe the evidence for a role of inflammation in the development of pulmonary fibrosis before discussing the recent controversy and the proposed alternative hypothesis.

1.3.1 Evidence to support the role of inflammation in the development of pulmonary fibrosis

Histological evaluation of lung tissue from patients with pulmonary fibrosis shows clear evidence of inflammation. Patients also show increased inflammatory cell numbers in BAL fluid, where both neutrophils and eosinophils have been described to be present in increased numbers (Reynolds *et al.,* 1997). BAL fluid neutrophilia and evidence of neutrophils in lung tissue have been demonstrated in patients with IPF (The BAL Cooperative Group Steering Committee, 1990), and an increase in the percentage of neutrophils or eosinophils (>5%) has generally been associated with a worse prognosis, with a higher likelihood of disease progression and a failure to respond to immunsupression (Turner-Warwick and Haslam, 1987; Hallgren *et al.,* 1987; Schwartz *et al.,* 1994). In contrast, lymphocytosis (>20%) on initial BAL occurs in <20% patients but appears to be associated with longer survival and a better response to therapy (Turner-Warwick and Haslam, 1987; Hallgren *et al.,* 1987; Schwartz *et al.,* 1994).

Studies have also shown inflammatory cells to be activated, releasing a variety of products which could cause tissue injury. Neutrophils have the capacity to release oxidants, proteases, complement fragments, arachidonic acid metabolites, and various cytokines which injure lung parenchymal cells. But the signals that recruit neutrophils to the lung, and perpetuate neutrophillic alveolitis are not yet fully understood. Leukocytes can be attracted into the lung by chemoattractants released both directly from alveolar macrophages and those released from lung parenchymal cells. The stimulus for the release of chemoattractants by lung parenchymal cells is at least in part, due to cytokines generated from alveolar macrophages and other cellular sources including alveolar epithelial cells, fibroblasts, T-helper cells and mast cells. This provides a self-perpetuating loop for continued inflammation.

The most commonly studied of these pro-inflammatory mediators are the early response cytokines TNF-α and IL-1 which have both been shown to be elevated in patients with pulmonary fibrosis (Zhang *et al.,* 1993). TNF-α and IL-1 are both induced early after injury, and lead to pain, fever, and vasodilatation, (reviewed by Dinarello, 2000). Both

cytokines also stimulate the production of lipid mediators, nitric oxide, chemokines, and stimulate fibroblast proliferation (Singh *et al.,* 1988; Vilcek *et al.*, 1986; Schmidt *et al.,* 1982). IL-1β has also been shown to stimulate fibroblast collagen deposition (Singh *et al.,* 1988; Postlethwaite *et al.,* 1988, see **section 1.4.4**).

The IL-8 family of cytokines (or chemokines) are another group which have been studied extensively. They represent at least 18 highly reactive peptides, whose biological activities include chemotactic activity for leukocytes (neutrophils, eosinophils, monocytes, lymphocytes), angiogenic activity, induction of collagen synthesis and cell proliferation. IL-8 is known to be chemotactic for neutrophils and increased levels of IL-8 in patients with IPF correlate with increased numbers of neutrophils in BALF from these patients (Carre *et al.,* 1994; Southcott *et al.,* 1995).

Animal models of pulmonary fibrosis have been a useful tool to study the role of pro-inflammatory mediators in the process. In the bleomycin model of fibrosis (see **Chapter 2**) an acute inflammatory response is seen in the first 7 days which precedes matrix deposition and fibrosis characterised by an increase in lung collagen content 14-21 days following instillation of bleomycin. The intensity of inflammation has been directly correlated with the intensity of fibrosis in a rabbit model (Shen *et al.,* 1988) and the list of pro-inflammatory mediators which increase following administration of bleomycin is extensive. Gene knockout and transgenic mice, along with systems which locally over-express specific genes have helped to further understand the biology of the inflammatory response in this model. For example, TNF-α receptor knockout mice are protected from fibrosis induced by asbestos inhalation (Liu *et al.,* 1998) and neutralising antibodies to TNF-α block silica-induced pulmonary fibrosis in mice (Piguet *et al.,* 1993b). Conversely, following over-expression of TNF-α cDNA in rat lung through adenoviral transfer, Sime and colleagues demonstrated severe pulmonary inflammation and fibrosis (Sime *et al.,* 1998). Similarly, the transient over-expression of IL-1β using adenoviral gene transfer was shown to lead to extensive fibrosis following severe inflammation (Kolb *et al.,* 2001), and conversely, a study using an IL-1 receptor antagonist was shown to prevent fibrosis in both silica- and bleomycin-induced mouse models (Piguet *et al.,* 1993b).

The role of T helper (Th) cells in the inflammatory response observed in pulmonary fibrosis is well studied, specifically the cytokine profiles secreted by these cells which

can be classified as either Th1 or Th2. Th1 cytokines include IFN-γ, IL-2, IL-12, IL-18, and Th2 IL-4, IL-5, IL-6, IL-10 and IL-13 (Mosmann *et al.,* 1986; Cher and Mosmann, 1987). Studies have suggested that an imbalance in the expression of Th1:Th2 cytokines may be important in the pathogenesis of disorders with an inflammatory component such as asthma, systemic sclerosis and IPF, where Th2 responses mediate allergic inflammation and chronic fibroproliferation (Hoyle and Brody, 2001, Lukacs *et al.,* 2001). Using in situ hybridisation and immunohistochemistry, sections of lung tissue taken from patients with IPF have been examined for the presence of a Th1 vs Th2 cytokine imbalance. This showed the predominance of Th2 over Th1 (Wallace *et al.,* 1995) and supports the hypothesis that a persistent inbalance in the expression of Th1:Th2 cytokines may be a mechanism for the progression of pulmonary fibrosis.

IFN-γ is the prototypic Th1 cytokine and the other Th1 cytokines IL-12 and IL-18 appear to act by inducing IFN-γ (Okamura *et al.,* 1998). It has several potential antifibrotic actions and has been shown to inhibit fibroblast proliferation and collagen synthesis *in vitro* (Jimenez *et al,* 1984; Gurujeyalakshmi and Giri, 1995, see **section 1.4.4**) and antagonises the functions of the pro-fibrotic mediator TGF-β (Gurujeyalakshmi and Giri, 1995; Eickelberg *et al.,* 2001). *In vivo,* repeated administration of IFN-γ has been shown to reduce ECM accumulation using the bleomycin model of pulmonary fibrosis in mice (Giri *et al.,* 1986; Granstein *et al.,* 1987; Hyde and Giri, 1990). However, in contrast, a study published in 2001 described reduced inflammation, weight loss and mortality in IFN-γ deficient mice compared with wild type controls, which was accompanied by reduced levels of lung collagen production 21 days following administration of bleomycin (Chen *et al.,* 2001). Despite this contrasting *in vivo* data, an open randomised trial of 18 patients with IPF found that repeated administration of IFN-γ together with a low dose of the steroid prednisolone was superior to prednisolone alone in preserving lung function in patients with mild to moderate IPF (Ziesche *et al,* 1999). Currently, the follow-up large, multicentre, randomised, double-blind, placebo-controlled, phase III study of the safety and efficacy of IFN-γ-1b is being completed. Preliminary analysis of these data suggest that although IFN-γ produced no differences in the observed rate of progression-free survival, it did appear to decrease mortality, primarily in patients with mild to moderate disease (Raghu *et al.,* 2003).

1.3.2 Evidence to suggest that inflammation is not crucial for the development of pulmonary fibrosis

The dogma that fibrogenesis is always preceded by, and potentially perpetuated by an early inflammatory response has been recently challenged in the context of IPF. This debate stems from the inadequacy of the current treatment for IPF where corticosteroids have been used for 50 years with little evidence to suggest that anti-inflammatory therapy is beneficial in the majority of patients with IPF (see **section 1.1.3**). Diseases involving a significant inflammatory component such as sarcoidosis and hypersensitive pneumonitis respond better to corticosteroid therapy and following the ERS/ATS reclassification of the idiopathic interstitial pneumonias (IIPs, described in **section 1.2**), so too do the inflammatory forms of IIP such as NSIP or DIP when compared with the more 'fibrotic' form of UIP (Bjoraker *et al.,* 1998; Daniil *et al.*, 1999).

Several groups including Selman and Pardo (Selman and Pardo, 2002), and Gauldie (Gauldie, 2002a; Gauldie *et al.,* 2002b), have developed an alternative hypothesis in which they suggest that rather than the traditional idea of an early inflammatory response leading to a late fibrotic stage, IPF is a fibroproliferative disorder preceded by epithelial cell activation leading to aberrant tissue remodeling, with inflammation appearing only after the development of fibroblastic foci. They suggest that the chronic and progressive nature of IPF is therefore driven by the alteration of mesenchymal and epithelial cell phenotypes by cytokines and matrix, rather than any inflammatory component. Katzenstein and Myers previously hypothesised that fibrosis can occur in the absence of inflammation; in a histological study, they showed IPF to be characterised by a pattern of UIP where the inflammatory component occurs mainly in areas of collagen deposition (fibroblastic foci) or honeycomb change, and rarely involves otherwise unaltered alveolar septa (Katzenstein and Myers, 1998).

This concept is also supported by several *in vivo* studies using adenovirus vectors to overexpress individual cytokine genes in the lung epithelium. These studies suggest that remodelling and fibrosis can occur in an animal model either subsequently to acute inflammation, as seen with over-expression of IL-1β (Kolb *et al.,* 2001), or independently of inflammation, as seen with the over-expression of active TGF-β_1 (Sime *et al.,* 1997). Furthermore, over-expression of IL-1β resulted in induction of TGF-β in addition to acute inflammation, and fibrosis occurred only after the transient expression of IL-1β has subsided, but the induced levels of TGF-β were sustained (Kolb

et al., 2001; Sheppard, 2001), suggesting that overexpression of TGF-β, and not the acute inflammation, is essential for the progression of fibrogenesis.

However, these recent discussions are specifically aimed at IPF as a disease and the authors do not claim that these concerns over the role of inflammation in the development of fibrosis are applicable in all processes which lead to the end point of pulmonary fibrosis.

In summary, the role of inflammation in the development of fibrosis, particularly in IPF currently remains controversial. However, it is clear that in patients inflammation is present in tissue taken from diseased lung, and increased inflammatory cell numbers have been shown to be present in BALF. Animal models further highlight the importance of the inflammatory response in the development of bleomycin induced pulmonary fibrosis but questions remain as to how closely the animal models available mimic human disease. *In vivo* strategies which block inflammation have been shown to block the development of fibrosis to varying degrees although this does not translate to human disease in the use of corticosteroids. Current ongoing studies using the antiinflammatory immunomodulator IFN-γ may provide hope for the future treatment of the process, however further research is needed to understand the role of inflammation in the development of fibrosis.

Where questions may arise over the importance of the inflammatory response in the development of fibrosis, there is no such doubt over the role of matrix deposition in the process. Increased levels of extracellular matrix proteins in the lung, particularly collagens, are the key feature of the disease, and as a direct result gas exchange in the lung is severely compromised in the affected individual. The following section will specifically discuss collagen distribution and metabolism in both the normal and fibrotic lung, along with the myofibroblast, the major producer of collagen in the fibrotic lung, and mediators which affect its production.

1.4 Extracellular matrix in the lung

The extracellular matrix (ECM) in the lung, like other organs, is an intricate network of macromolecules composed of a variety of versatile proteins and polysaccharides including the collagens, elastic fibres, proteoglycans, fibronectin and laminin. The

macromolecules are secreted locally and assembled into organised meshworks in close association with the surface of the cell which produced them. The primary role of the ECM is to provide a scaffolding to stabilise the physical structure of the lung, whilst maintaining flexibility required for respiration. However, the ECM is not inert, but plays an active role in regulating the behaviour of the cells in contact with it, influencing their development, morphology, migration, proliferation, and function, both physiologically and in pathological conditions.

Collagen and elastin fibres are embedded in a highly hydrated, gel-like substance composed of proteoglycan molecules (reviewed by Wight *et al.,* 1991). The polysaccharide gel resists compressive forces on the matrix whereas collagen fibres strengthen and help organise the ECM, conferring tensile strength to the alveolar walls (reviewed by McAnulty and Laurent, 1995 and Chambers and Laurent, 1997). Rubber-like elastin fibres (reviewed by Clearly and Gibson, 1996; Mariani *et al.*, 1997) give the matrix resilience and the ability to stretch and recoil throughout respiration without damage to the lungs and are partially concentrated in the parenchymal regions where together with collagen, elastin provides the structural skeleton of the alveolar unit (Burri, 1985). The cell adhesive proteins such as fibronectin and laminin are also a key component of the ECM in the lung. Fibronectin is present throughout the interstitium whilst laminin is localised to the alveolar wall and basement membranes (Sethi *et al.,* 1999). Both proteins help in cell attachment and spreading through their interactions with integrins (reviewed by Juliano and Haskill, 1993); fibronectin promotes the attachment of fibroblasts and other cells to the matrix, whilst laminin promotes the attachment of epithelial cells to the basal lamina.

However, the collagen superfamily represents the most abundant group of proteins in the lung and excessive deposition of extracellular matrix proteins, particularly the collagens, is inarguably the primary characteristic of pulmonary fibrosis. Therefore the following section will concentrate on the collagen superfamily, discussing structure, metabolism and function in the normal lung, before discussing how this is altered in the fibrotic lung.

1.4.1 Collagen distribution and metabolism in normal lung

At least 27 members of the collagen superfamily have been described to date (Pace *et al.,* 2003), each with specific mechanical and biochemical properties. However, they all

share a basic structure of three alpha (α)–polypeptide chains coiled into a triple helix. Over 40 distinct α-chains have been described which combine in various ways to form the 11 collagens identified in the lung (Pace *et al.,* 2003; Boot-Handford *et al.,* 2003). For example, collagen III is a homodimer consisting of three α1(III)-chains, whereas collagen I is a heterodimer consisting of an α2(I)-chain combined with two α1(I)-chains. Individual α-chains contain a large proportion of the smallest amino acid, glycine which allows close packing and hydrogen bonding between the chains. The generalised structure of an α-chain is $(Gly\text{-}X\text{-}Y)_n$, where the X and Y positions are frequently held by the imino acids proline and hydroxyproline. Hydroxyproline is not incorporated directly into proteins, but is produced post-translationally via the hydroxylation of proline. This creates extra hydrogen bonds, which further stabilise the triple helix. Although the incorporation of amino acids other than proline and hydroxyproline decrease stability of the triple helix, they are essential for the assembly of the triple helical molecules into fibrils.

The fibrillar collagens (I, II, III, V and XI) contain approximately 1000 amino acids in the (Gly-X-Y) formation with 2 short terminal domains. Approximately 90% of collagen in the lung is fibrillar types I and III in a ratio of 2:1 (Kirk *et al.,* 1984). These are distributed throughout the interstitium, large bronchi and blood vessels and provide strength, rigidity (collagen I) and compliance (collagen III) to tissues (Mays *et al.,* 1988). Fibroblasts are the major producer of these collagens (Hance *et al.,* 1976), but they may be synthesised by other cells, such as endothelial cells, epithelial cells, alveolar type II cells and smooth muscle cells. Non-fibrillar collagens (including IV, VI and VII) contain other residues interspersed within their α-chains. Type IV collagen is the major component of the alveolar and capillary basement membranes (Crouch *et al.,* 1990) and the most common non-fibrillar collagen in the lung. The short-non-triple-helical sequences interspersed within the α-chains provide increased flexibility to collagen IV.

The collagens exist in a dynamic equilibrium involving constant degradation and synthesis of new molecules. Synthesis of collagen fibrils comprises firstly of intracellular steps to assemble and secrete procollagen, a longer molecule containing additional propeptides at both the N- and C-terminal ends. Secondly, the procollagen is converted into collagen close to the cell membrane, and is then incorporated into stable,

cross-linked collagen fibrils. Briefly, procollagen DNA is transcribed to pre-mRNA in the nucleus and is subsequently spliced to form functional mRNA. Translation occurs in ribosomes of the rough endoplasmic reticulum (RER) to make pre-procollagen α-chain. The pre-procollagen α-chain contains an N-terminal signal peptide that facilitates movement across the RER and is cleaved either during translation or shortly after peptide synthesis to form the procollagen α-chain. Whilst the α–chains are growing on ribosomes, co- and post-translational modifications occur including hydroxylation of specific proline and lysine residues in the Y position to form hydroxyproline and hydroxylysine. Mannose-rich oligosaccharides are transferred onto aspargine-linked carbohydrate units in the C-propeptides of fibrillar collagens and some domains in non-fibrillar collagens.

In fibrillar collagens, triple helix formation occurs from the C-terminus to the N-terminus following alignment of 3 procollagen α-chains which form both intra- and interchain disulphide bonds between lysine and hydroxylysine residues. The helical procollagen molecules are then transported to the Golgi where they are packaged in secretory vesicles for transport into the extracellular space. During or immediately after secretion, the N- and C-terminal propeptides are enzymatically cleaved by procollagen N- and C-proteinase respectively. In fibrillar collagens, cleavage of propeptides leads to spontaneous assembly into fibrils at the cell plasma membrane. The fibrils are initially held together by electrostatic interaction, however following cross-linking by lysyl oxidase forming strong covalent bonds, collagen fibrils possess the tensile strength of steel. Most non-fibrillar collagens usually undergo proteolytic processing but covalently cross-link with other molecules to form open-meshed networks.

Collagen synthesis and degradation continues in the lung throughout life and turnover rates for newly synthesised collagen are extremely rapid. *In vivo* it has been estimated that an amount of collagen equivalent to one tenth of the total collagen content is synthesised and degraded each day (Laurent 1982; McAnulty and Laurent, 1987). *In vitro,* lung fibroblasts have been shown to degrade between 10-40% of the procollagen they synthesise depending on the culture conditions (reviewed by Rennard *et al.,* 1982). This process has been shown to occur within 15 mins *in vivo* (McAnulty and Laurent, 1987) and is likely to occur intracellularly as 15 minutes is considerably less than that required by cells to secrete collagen molecules (Dehm and Prockop, 1972, 1973; Grant *et al.,* 1972). In the lung, the proportion of newly synthesised procollagen degraded

ranges between 30% in young animals to 80% in aged animals (Mays *et al.,* 1989). Degradation of defective procollagen molecules occurs in lysosomes with enzymes such as cathepsins B, D, and L being capable of degrading nonhelical procollagen molecules. Triple helical protein molecules are degraded through an alternative pathway termed the 'basal degradation' pathway and it has been proposed that this occurs in the trans-Golgi apparatus (Barile *et al.,* 1990). Extracellular degradation is much slower as the collagen fibril is extremely stable. Degradation occurs by proteases released by a number of cell types including inflammatory and mesenchymal cells. These include the matrix metalloproteases (MMPs); the collagenases, stromelysins and gelatinases, and the serine proteases.

1.4.2 Collagen distribution and metabolism in fibrotic lung

In the normal lung alveolar walls are thin to allow maximal gaseous exchange, and contain small amounts of collagen for structural support. However, in pulmonary fibrosis excess collagen deposition leads to thickening of the interstitium and loss of lung architecture resulting in impaired lung function. Excess collagen deposition in the lungs of patients with pulmonary fibrosis results in small, stiff lungs which show radiographic honeycombing. Many studies have aimed to establish the cause of excess deposition of collagen in pulmonary fibrosis. There is evidence to suggest that the balance between synthesis and degradation of collagen is altered in pulmonary fibrosis leading to increased deposition of collagen in the lung (Laurent, 1987). This may be caused by direct dysregulation of collagen synthesis and/or degradation i.e., collagen gene expression, or indirectly through enhanced proliferation of fibroblasts, or changes in levels of both pro- and anti-fibrotic soluble mediators which affect collagen synthesis and/or degradation. Mediators which affect fibroblast function will be discussed further in **section 1.4.4**.

In human disease, BALF procollagen peptide levels have been shown to be increased in CFA (Low *et al.,* 1983; Cantin *et al.,* 1988; Bjermer *et al.,* 1989), systemic sclerosis (Harrison *et al.,* 1990) and sarcoidosis (Low *et al.,* 1983; Bjermer *et al.,* 1986). Using in situ hybridisation, type I procollagen mRNA expression has also been shown to be increased in human lung (Broekelmann *et al.,* 1991). Furthermore, an increase in C-terminal procollagen peptide has also been demonstrated by immunohistochemistry (McDonald *et al.,* 1986) and levels of procollagen C- proteinase, the enzyme which

cleaves the C-terminal procollagen peptide, has been shown to be enhanced in patients with ARDS (Entzian *et al.,* 1990).

Using the bleomycin model *in vivo*, rates of collagen synthesis have also been shown to be increased (Phan *et al.,* 1981; Laurent and McAnulty, 1983) in association with increased levels of type I and III collagen mRNAs (Kelly *et al.,* 1985; Raghow *et al.,* 1985; Hoyt and Lazo, 1988; Shahzeidi *et al.,* 1993) Furthermore, lung fibroblasts obtained from rats treated with bleomycin show enhanced collagen synthesis compared with control fibroblasts (Phan *et al.,* 1985). Microarray technology has recently demonstrated increased expression of the genes encoding for collagens I and III in human fibrotic tissue samples compared with normal lung and expression of genes encoding collagen VI, tenascin C, osteopontin and fibronectin were also increased (Zuo *et al.,* 2002).

Collagen degradation is less well studied in human pulmonary fibrosis but increased levels of collagenases have been reported in BALF of patients with CFA (Gadek *et al.,* 1979). However, other studies have suggested that the increase in collagen content in CFA is related to a decrease in collagenase activity (Selman *et al.,* 1986). MMPs degrade components of the ECM, and Selman and co-workers (Selman, *et. al.,* 2000) recently evaluated the expression of the collagenase subfamily (MMP-1, -9 and –13), the gelatinase subfamily (MMP-2, 9), membrane type-1 MMP and all 4 of the tissue inhibitors of metalloproteases (TIMPS) in biopsies taken from patients with CFA. This study showed that there was a higher expression of TIMPS compared with MMPs in the fibrotic lung. Overall, the study concluded that there is a prevailing non-degradative microenvironment in the lungs of patients with CFA.

1.4.3 The myofibroblast

The major producers of extracellular matrix macromolecules in the active fibrotic lesion, the fibroblastic foci, are the fibroblasts, and any change in their numbers or function will undoubtedly effect the function of the lung as an organ. Fibroblastic foci include a heterogeneous population of fibroblasts with an intermediate phenotype of a fibroblast and a smooth muscle cell, termed the myofibroblast. These cells express α-smooth muscle actin (α-SMA), suggesting the fibroblasts have acquired morphological and biochemical features of contractile cells (Adler *et al.* 1989; Mitchell *et al.,* 1989; Zhang *et al.,* 1997; Zhang *et al.,* 1996; Desmouliere and Gabbiani, 1995). The presence

of myofibroblasts in both patients with, and animal models of, pulmonary fibrosis is well documented (Adler *et al.,* 1989; Mitchell *et al.,* 1989; Kuhn and McDonald, 1991; Pache *et al.,* 1998), and the failure of the disease to resolve in patients with progressive disease, correlates with the persistence of myofibroblasts (Kuhn and McDonald, 1991).

Myofibroblasts have been identified as the predominant source of increased collagen gene expression in the fibrotic lung (Zhang *et al.,* 1994), and their contractile phenotype has been shown to correlate with the development of decreased lung compliance (Alder *et al.,* 1989; Mitchell *et al.,* 1989; Zhang *et al.,* 1994). These cells have also been shown to be a significant source of pro-inflammatory and pro-fibrotic mediators such as TGF-β_1 (Zhang *et al.,* 1995). These mediators themselves recruit inflammatory cells, creating a positive feedback loop intensifying, or prolonging the inflammation associated with the fibrotic foci (Phan, 2002).

Fibroblasts transition into myofibroblasts occurs though a process regulated by cytokines and ECM components (Desmouliere and Gabbiani, 1995), of which TGF-β is thought to be the most efficient (Desmouliere, 1995). TGF-β_1 has been shown to stimulate α-SMA mRNA and protein production in myofibroblasts (Mitchell *et al.,* 1989; Zhang *et al.,* 1996; Desmouliere *et al.*, 1993) and this process has been shown to be inhibited by both IFN-γ and PGE_2 (Yokozeki *et al.,* 1999; Kolodsick *et al.,* 2003), however the mechanisms by which myofibroblast transition is regulated are still not fully elucidated.

1.4.4 Mediators of fibroblast function

In addition to regulating fibroblast-myofibroblast transition, soluble mediators such as cytokines are important in regulating the other key features of pulmonary fibrosis such as fibroblast proliferation and collagen production. It is generally accepted that an important underlying molecular mechanism in the pathogenesis of pulmonary fibrosis is the over- and/or under-production and dysregulation of certain cytokines and polypeptide growth factors (reviewed by Coker *et al.,* 1998; Zhang and Phan, 1996; Smith *et al.,* 1995; Khalil and Greenberg, 1991) which regulate fibroblast function at various levels.

For example, collagen production is regulated at the level of synthesis, deposition and turnover by altering the levels of gene transcription, mRNA processing and degradation

(reviewed by Ramirez and DiLiberto, 1990; McAnulty and Laurent, 1995). The list of potentially important mediators is extensive and whilst this thesis can not discuss each important mediator in turn, **Table 1.1** summarises those mediators known to have stimulatory effects on fibroblast collagen production and proliferation, whereas **Table 1.2** summarises those mediators known to have negative regulatory effects.

Mediators which increase fibroblast collagen production		Mediators which increase fibroblast proliferation	
Angiotensin II	Brilla *et al.*, 1994	Angiotensin II	Marshall *et al.*, 2000
CTGF	Frazier *et al.*, 1996	CTGF	Frazier *et al.*, 1996
ET-1	Dawes *et al.*, 1996	ET-1	Peacock *et al.*, 1992
Insulin	Goldstein *et al.*, 1989	EGF	Kurata and Hata, 1991
Insulin-like-growth factor (IGF)	Goldstein *et al.*, 1989	Fibroblast activating factor	Wahl and Gately, 1983
IL-1	Postlethwaite *et al.*, 1988	Fibronectin	Bitterman *et al.*, 1983
IL-4	Postlethwaite *et al.*, 1992	IGF	Phillips *et al.*, 1987
IL-6	Duncan and Berman, 1991	IL-1	Singh *et al.*, 1988
TGF-β	Varga and Jimenez, 1986	IL-4	Trautmann *et al.*, 1998
Thrombin	Chambers *et al.*, 1998	IL-13	Kraft *et al.*, 2001
		PDGF	Oliver *et al.*, 1989
		Thrombin	Gray *et al.*, 1990
		TNF-α	Vilcek *et al.*, 1986

Table 1.1 Mediators known to promote fibroblast collagen production and proliferation

Mediators which decrease fibroblast collagen production		Mediators which decrease fibroblast proliferation	
EGF	Kurata and Hata 1991	IFN-α	Elias *et al.*, 1987
IFN-α	Jimenez *et al.*, 1984	IFN-β	Tominaga and Lengyel 1985
IFN-γ	Heckmann *et al*, 1989	IFN-γ	Elias *et al.*, 1987
PGE_2	Saltzman *et al.*, 1982	PGE_2	Oliver *et al.*, 1989
Retinoic acid	Oikarinen *et al.*, 1985		

Table 1.2 Mediators known to inhibit fibroblast collagen production and proliferation

In addition to its role in fibroblast-myofibroblast transition, one of the most important pro-fibrotic mediators listed in **Table 1.1** is transforming growth factor-beta (TGF-β). This thesis is primarily concerned with the role of the negative regulatory mediator prostaglandin E_2 (PGE_2, **Table 1.2**) which is an important negative regulator of the

actions of TGF-β. The following sections will first further discuss the role of TGF-β in the development of the fibrotic response before introducing PGE_2.

1.4.5 Transforming growth factor-beta (TGF-β)

In mammals there are three isoforms of TGF-β (TGF-β_{1-3}) all of which signal through the same receptors and have similar biological functions. However, it is TGF-β_1 which has been the most widely studied in the context of fibrogenesis and the continued overproduction of TGF-β_1 appears to be a central factor in the molecular mechanisms which lead to pulmonary fibrosis.

TGF-β_1 is a fibroblast chemoattractant and is able to exert a bimodal effect on fibroblast proliferation via an autocrine PDGF-dependent pathway (Battergay *et al.,* 1990). Furthermore it has been shown to stimulate biosynthesis of type I collagen (Roberts *et al.,* 1986; Ignotz and Massague, 1986; Varga and Jimenez, 1986) and is the most potent stimulator of fibroblast collagen production described to date. The stimulatory effect of TGF-β_1 on fibroblast collagen production is mediated both transcriptionally and translationally; TGF-β_1 is known to increase collagen mRNA transcription and stability (Ignotz *et al.,* 1987; Raghow *et al.,* 1987; Varga *et al.,* 1987). Furthermore, TGF-β_1 has also been shown to reduce the proportion of newly synthesised procollagen degraded before secretion (McAnulty *et al.,* 1991), and to limit extracellular degradation of collagen through inhibiting the expression of collagenases, whilst promoting expression of their respective inhibitors (Overall *et al.,* 1989; Shi *et al.,* 1990; Edwards *et al.,* 1987). In addition to collagen, TGF-β_1 is a potent modulator of a number of genes known to be involved in tissue remodelling and fibrosis. For example, TGF-β_1 increases the production and/or activity of connective tissue growth factor (CTGF, Lasky *et al.,* 1998), and other genes of the ECM: fibronectin (Ignotz and Massague, 1986) and proteoglycans (Bassols and Massague, 1988), which together result in an excess accumulation of ECM proteins. Beside the lungs, the role of TGF-β has also been demonstrated in other organs undergoing fibrotic changes (Khalil *et al.,* 1991).

In patients with IPF, immunohistochemical studies have shown enhanced expression of TGF-β_1 in a number of cell types including alveolar macrophages bronchiolar epithelial cells, fibroblasts and myofibroblasts (Khalil *et al.,* 1991, Zhang *et al.,* 1995). Furthermore, a large number of studies using animal models of pulmonary fibrosis have

confirmed both the fibrogenic nature of TGF-β_1 over-expression and have demonstrated the anti-fibrotic effects of TGF-β_1 inhibition, such as with anti-TGF-β_1 antibodies (reviewed by Lasky and Brody, 2000).

The pleiotrop ic actions of TGF-β_1, its important influence on ECM metabolism, and its ability to regulate the production of other cytokines in an a paracrine fashion (Lyons and Moses 1990), makes it arguably the most important pro-fibrotic mediator. However, the other side of the balance must be considered; not only is over-production of pro-fibrotic mediators important in the development of the disease, but so too is the under-production of potentially important anti-fibrotic mediators. One such mediator is prostaglandin E_2, or PGE_2. TGF-β_1 is one of the mediators known to induce the synthesis of PGE_2 which is a potent inhibitor of both fibroblast collagen production, proliferation and myofibroblast differentiation in the lung. It is this autocrine synthesis of PGE_2 that is responsible for the anti-proliferative effects of TGF-β_1 (McAnulty *et al.*, 1997) and limits the stimulation of collagen synthesis by TGF-β_1 (McAnulty *et al.*, 1995). This thesis will specifically focus on the role of PGE_2 as a mediator of pulmonary fibrosis and the next sections will describe the physiological and pathological roles of PGE_2 in the lung and its biosynthesis via the cyclooxygenase pathway.

1.5 Prostaglandin E_2

PGE_2 is an eicosanoid, a class of lipid mediators derived from phospholipase-released arachidonic acid. The eicosanoids are involved in numerous biological functions and include both prostanoids and leukotrienes, generated via the cyclooxygenase and lipoxygenase pathways respectively. There are currently two cyclooxygenase (COX) isoforms generated from separate genes, namely COX-1 and COX-2. Physiologically, prostaglandins are implicated in ovulation, fertilisation, platelet aggregation and maintenance of renal function, in addition to being regulators of fever, pain and inflammation. Briefly, those generated via the actions of COX-1 are seen to have a homeostatic function, whereas those generated via COX-2 are generally considered inflammatory and pathogenic, although this is definition not clear-cut. The COX isoforms will be discussed in greater detail in **sections 1.8-1.10**. Prostanoids generated by COX-1 and COX-2 include the prostaglandins (PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2) and thromboxane (TxA_2) and their biosynthetic pathway is shown in **Figure 1.1.**

Leukotrienes (LTB_4, LTC_4, LTD_4 and LTE_4) will not be discussed in significant detail in this thesis, however there is also substantial evidence for a role for leukotrienes in pulmonary fibrosis. They have been reported to directly stimulate fibroblast proliferation and collagen synthesis (Baud *et al.,* 1987) and this is consistent with the finding of a constitutive activation of 5-liopxygenase (5-LO) in the lungs of patients with IPF (Wilborn *et al.,* 1996). Lung homogenates from these patients contained 15-fold more LTB_4 than homogenates from non-fibrotic lungs (Wilborn *et al.,* 1996). Furthermore, mice deficient in 5-LO are protected from bleomycin-induced lung fibrosis (Peters-Golden *et al.,* 2001).

There is substantial evidence to suggest that PGE_2 may play an important role in the pathogenesis of pulmonary fibrosis, both directly via effects on fibroblast number and function, and indirectly by modulating production of pro-fibrotic mediators in the lung. The following sections will firstly discuss its biosynthesis via the cyclooxygenase pathway, followed by the effects of prostaglandins, specifically PGE_2, in the lung, and the cellular mechanisms by which it may modulate fibroblast function and the inflammatory response. Finally, evidence will be presented to suggest that levels of PGE_2, and cyclooxygenase-2 (COX-2), the rate-limiting enzyme in its biosynthesis, are altered in patients with pulmonary fibrosis.

1.6 Biosynthesis of PGE_2

The key enzyme in PGE_2 biosynthesis is cyclooxygenase (COX, also known as prostaglandin H_2 synthase, PGHS), which initially converts phospholipase-released arachidonic acid to prostaglandin G2 (PGG_2) by a cyclooxygenase reaction. COX also then catalyses the reduction of the 15-hydroperoxyl group of PGG_2 to prostaglandin H_2 (PGH_2) by a peroxidase reaction. PGH_2 is then converted by a hydroperoxidase reaction to the various prostaglandins; PGE_2 PGD_2 $PGF_{2\alpha}$ PGI_2 and thromboxane (TX), by tissue specific synthases. This pathway is shown in **Figure 1.1.** Two COX isoforms which are products of separate genes have been described to date; COX-1 and COX-2, and in 2002 a sequence for a proposed COX-3 was described. The COX isoforms are very similar in length; COX-1 has 576 amino acids and COX-2 604, of which 60-65% are in an identical sequence (O'Banion *et al.,* 1992; Ryseck *et al.,* 1992; Fletcher *et al.,* 1992; Hsi *et al.,* 1994).

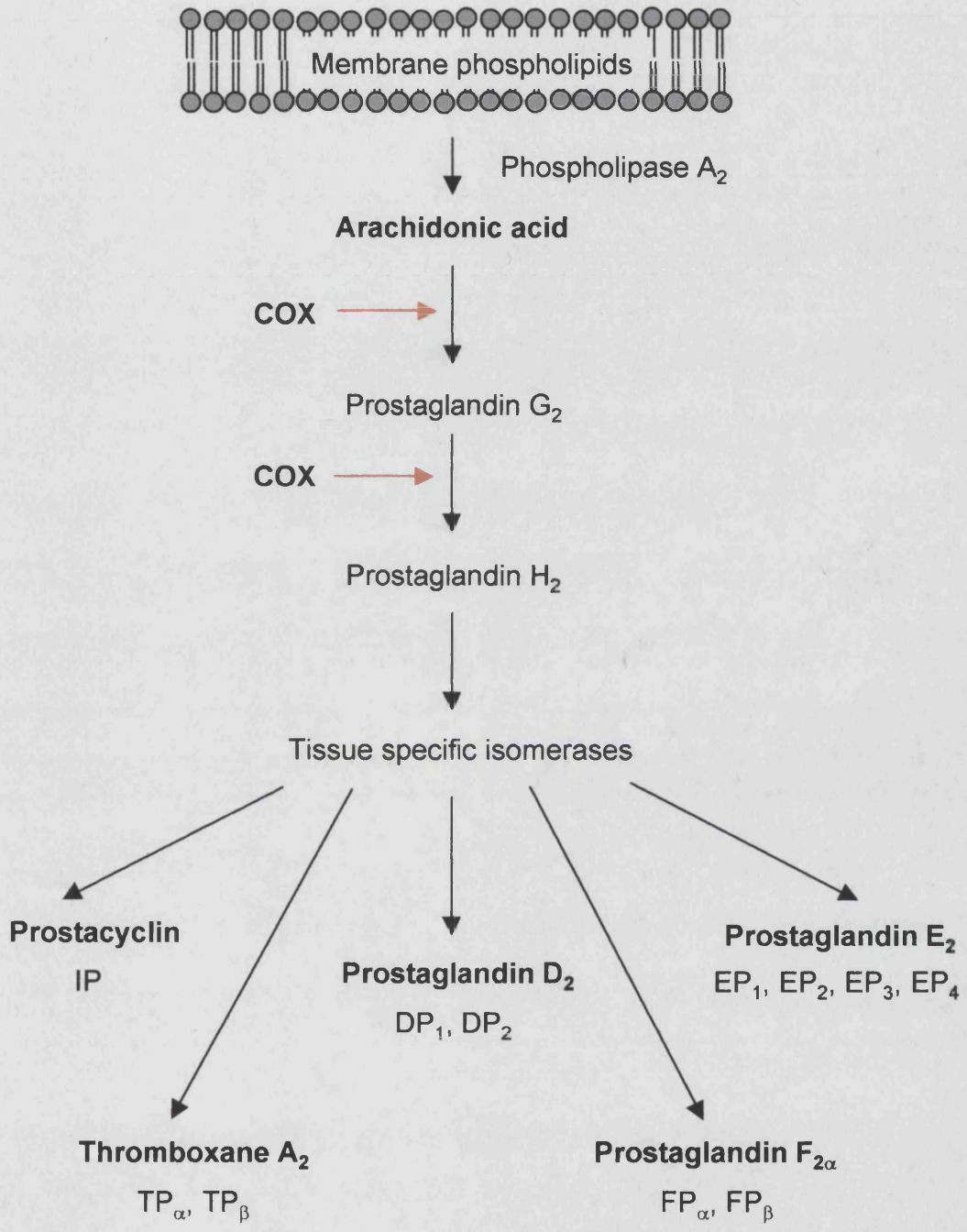


Figure 1.1 Prostanoid biosynthetic pathway
Schematic to show intermediate steps in biosynthesis of specific prostanoids formed by cyclooxygenase reaction and their specific receptors

The isoforms are similar in enzymatic function but their cell-specific expression and regulation, subcellular localisation, and their physiologic functions are thought to be quite different (Smith *et al.,* 1994). For example, whilst both COX-1 and COX-2 are present on the lumenal surfaces of the endoplasmic reticulum and of the inner and outer membranes of the nuclear envelope (Morita *et al.,* 1995, Spencer *et al.,* 1999), COX-2 is more highly concentrated in the nuclear envelope (Morita *et al.*, 1995). Generally the constitutive COX-1 is used in the immediate PG synthesis which occurs within several minutes of stimulation of Ca^{2+} mobilisers, whereas the inducible COX-2 is an absolute requirement for delayed PG synthesis, which lasts for several hours after pro-inflammatory stimuli. COX-2 also has a primary immediate response after Ca^{2+} mobilisation.

The following sections will discuss the biosynthesis of PGE_2 in chronological order, beginning with substrate release, then discussing the COX isoforms, describing gene structure, function, activity, and roles, both physiological and pathological. PG synthases and receptors will then be discussed followed by the degradation pathway and pharmacological inhibitors of COX activity.

1.7 Substrate availability

The first step in the production of prostanoids is the release of free arachidonic acid (20:4*n*-6), the main constituent of phospholipid membranes. Arachidonic acid is the principal substrate for prostanoid biosynthesis in mammalian cells, although both COX isoforms are capable of utilising additional substrates, but to differing abilities. COX-2 will accept a wider range of fatty acids as substrates than COX-1 (Otto and Smith, 1995).

AA exists esterified in cell membrane phospholipids, however, non-esterified AA is required as the substrate for eicosanoid biosynthesis. Following an appropriate trigger, one or more of the phospholipase systems is activated at the endoplasmic reticulum and nuclear envelope to cleave AA from the glycerol backbone of the lipid bilayer (Holtzman *et al.,* 1991). AA can be mobilised by several different phospholipase A_2s. Amongst them $cPLA_2$ and type IIA and type V secreted PLA_2 ($sPLA_2$) predominately contribute to prostaglandin production.

Specific coupling between COXs and particular PLA_2s subtypes have been proposed, i.e., coupling of $cPLA_2$ and COX-2 and $sPLA_2$ with COX-1 (Reddy and Herschman, 1997; Bingham *et al.,* 1996). However, co-expression studies have clearly demonstrated that both $cPLA_2$ and several $sPLA_2$ isozymes are capable of supplying AA to both COX-1 and COX-2 in the immediate responses and mainly to COX-2 in the delayed responses (Murakami *et al.,* 1999a; Murakami *et al.,* 1999b; Murakami *et al.,* 1999c; Murakami *et al.,* 2001). Additionally, studies using $cPLA_2$ null mice have shown $cPLA_2$ to be essential for both immediate and delayed phases of PG generation. (Uozumi *et al.,* 1997; Bonventre *et al.,* 1997). The actions of $sPLA_2$s in either phase appear to be cell type-specific and depend on their temporal expression, secretion process, and sorting into particular membrane microdomains (Murakami *et al.,* 1999b; Murakami *et al.,* 2001).

1.8 COX-1

Both COX-1 and the proposed COX-3 are encoded from a single gene on chromosome 9 (Funk *et al.,* 1991; Kraemer *et al.,* 1992; Chandrasekharan *et al.,* 2002). COX-1 was originally purified from sheep vesicular gland (Van der Ouderaa *et al.,* 1977) and the primary structure was identified by cDNA cloning (Merlie *et al.,* 1988; Yokoyama *et al.,* 1988; DeWitt and Smith, 1988). The COX-1 gene is 22kb in length, contains 11 exons (Yokoyama and Tanabe, 1989) and is expressed in lung fibroblasts as two mRNA transcripts of 5.5kb and 2.7kb (Diaz *et al.,* 1992). The complete amino acid sequence was determined by cloning and sequence analysis of human genomic DNA encoding for the enzyme, and in its processed form, the glycoprotein has 576 amino acids and exists as a 72kDa homodimer (Yokoyama and Tanabe; 1989; DeWitt *et al.,* 1990; Picot *et al.,* 1994).

The COX-1 gene is constitutively expressed in nearly all tissues and prostanoids synthesised via COX-1 are generally thought to be responsible for homeostatic functions such as gastric cytoprotection, regulation of renal blood flow, and platelet aggregation. There are several putative transcriptional regulatory elements in the promoter region, including two Sp1 motifs, two AP-2 sites, an NF-IL6 motif, and a GATA but like many 'housekeeping genes', COX-1 lacks a TATA box, a CAAT box, and is GC-rich (Kraemer *et al.,* 1992). However, preferential expression at high levels occurs in certain tissues and expression increases in cell lines undergoing differentiation

(reviewed by Morita, 2002). Induction of COX-1 without differentiation has also been reported in guinea pig gallbladder inflammation where enhanced bradykinin-stimulated prostaglandin release has been shown to be due to de novo synthesis of COX-1 protein (Bogar *et al.,* 1999).

The physiological roles of COX-1 have been deduced from the deleterious gastrointestinal side effects of NSAIDs which are thought to be associated with inhibition of basal COX-1 levels. This has lead to the subsequent development of COX-2 selective NSAIDs by the pharmaceutical industry. Surprisingly, mice deficient in COX-1 do not exhibit evidence of spontaneous gastric injury in the absence of an inflammatory stimulus despite a 99% reduction in gastric PGE_2 levels (Langenbach *et al.,*1995). However, it has been shown that COX-1 deficient mice are more susceptible to acute dextran-sodium sulphate (DSS)-induced colitis (Morteau *et al.,* 2000). Additionally COX-1 null mice also show reduced indomethacin-induced gastric ulceration, reduced platelet aggregation and a decreased inflammatory response to AA (Langenbach *et al.,*1995).

1.9 COX-2

In the early 1990's COX was demonstrated to exist as two distinct isoforms (Xie *et al.,* 1991; Hla *et al.,* 1992). The COX-2 gene is found on chromosome 1 (Tay *et al.,* 1994) and is significantly smaller than the COX-1 gene at 8.3kb (Hla *et al.,* 1992; Appleby *et al.,* 1994; Kosaka *et al.,* 1994). It contains 10 exons and is expressed as a single 4.4kb mRNA transcript in fibroblasts (Diaz *et al.,* 1998).The cDNA for COX-2 encodes a polypeptide that before cleavage of the signal sequence contains 604 amino acids that is 60-65% identical to that of the human COX-1 polypeptide. The main differences between the genes are firstly that the first intron in COX-1 is lost in COX-2 and secondly, that the introns in the COX-2 gene are shorter than those in the COX-1 gene.

As a primary response gene, levels of COX-2 mRNA and protein are normally undetectable in most tissues, but are readily induced by a variety of mediators including pro-inflammatory and pro-fibrotic cytokines such as IL-1, TNF-α, or LPS, and hormones or oncogenes (Smith and Dewitt, 1996; Smith *et al.,* 1996; Herschman, 1996). Products of the COX-2 pathway themselves may also alter the expression of the enzyme; PGE_2 has been shown to upregulate COX-2 expression via upregulation of

cAMP levels in a variety of cell types including murine macrophages (Hinz *et al.*, 2000b) and human blood monocytes (Hinz *et al.*, 2000a). Expression of COX-2 also occurs in specialised tissues where it plays specific roles in individual processes such as reproduction (Lim *et al.*, 1999; Lim *et al.*, 1997), immunity (Rocca *et al.*, 1999) renal physiology (Cheng *et al.*, 1999) or pancreatic secretion (Robertson *et al.*, 1998). Factors which regulate COX-2 expression in these cells are often specific to the process, for example in the kidney, expression of COX-2 in the macula densa depends on lumenal salt concentrations (Harris *et al.*, 1994). The lung is the second highest source of COX-2 mRNA (O'Neill and Ford Hutchinson, 1993), and high levels of expression are associated with epithelial cells (O'Neill and Ford Hutchinson, 1993; Asano *et al.*, 1996) and macrophages (O'Neill and Ford Hutchinson, 1993).

COX-2 expression is controlled both at the level of gene transcription and post-transcriptional events. COX-2 is an inducible immediate-early gene and sequence analysis of the 5'-flanking region of the mouse (Fletcher *et al.*, 1992), rat (Sirois *et al* 1993) and human (Appleby *et al.*, 1994; Tazawa *et al.*, 1994; Kosaka *at al*, 1994) genes have shown several potential transcriptional regulatory elements, including a TATA box, a cyclic AMP response element (CRE), two NF-IL6 (C/EBP) motifs, two AP-2 sites, three Sp1 sites, two NF-kB sites and an E-box, allowing transcriptional regulation of COX-2 by a broad spectrum of mediators (Appleby *et al.*, 1994, reviewed by Tanabe and Tohnai, 2002). Since many activators, under different conditions, lead to regulation of COX-2 gene expression it is therefore difficult to identify a common signalling pathway in all cases. However, pathways common to the inflammatory response have been linked to the increased expression of COX-2 including the NFkB and NF-IL6 (C/EBP) pathways, as well as one or all of the three mitogen-activated protein kinase (MAPK) signalling pathways: ERK1/2, JNK/SAPK and p38RK/Mpk2 (Su *et al.*, 1996).

Post-transcriptionally COX-2 may also be regulated by affecting mRNA stability as it is highly unstable due to the presence of AU rich elements in the 3'UTR of the COX-2 gene (Appleby *et al.*, 1994; Dixon *et al.*, 2000). It has been reported that the MAPK signalling pathway is involved in regulating gene expression at the post-transcriptional level (Chen *et al.*, 1998; Winzen *et al.*, 1999) as activation of one or more MAPKs results in stabilisation of the target mRNA. Mediators which induce COX-2 gene expression such as TGF-β and IL-1α also stabilise COX-2 mRNA via induction of MAPK p38 (Diaz *et al.*, 1998; Gou *et al.*, 1998).

Anti-inflammatory cytokines such as IL-4, IL-10 and IL-13 have been reported to inhibit the expression of COX-2 (Lee, *et al.*, 1992; Onoe *et al.*, 1996; Niiro *et al.*, 1997), and despite the absence of a glucocorticoid response element (GRE) on the 5'-flanking region of the human COX-2 gene glucocorticoids, such as dexamethasone, strongly suppressed COX-2 expression induced by inflammatory stimuli. The mechanism of glucocorticoid-mediated repression of COX-2 gene expression involves suppression of AP-1 and NF-κβ-dependent transcription (Yang Yen *et al.*, 1990; Auphan *et al.*, 1995), although at 6 hours following treatment with dexamethasone, COX-2 transcription is only inhibited by 40% but COX-2 protein and mRNA are completely repressed, indicating the presence of other post-translational methods of repression (Newton *et al.*, 1998). Indeed, dexamethasone shortens the poly-A tail which confers stability to mRNA transcripts, resulting in the premature degradation of COX-2 mRNA (Ristimaki *et al.*, 1994; Newton *et al.*, 1998).

In summary, COX-2 has been shown to play a role in physiological processes such as renin secretion in the kidney, wound/ulcer healing, female reproductive functions, bone metabolism and vascular protection. The co-ordination of COX-2 gene expression occurs through a combination of regulating gene expression and post-translational events and this high level of complexity is needed to tightly control the expression of COX-2 which can have pathogenic effects when expression is dysregulated. Increased COX-2 induction has been primarily associated with a number of pathological processes such as acute and chronic inflammatory states, pain, fever, Alzheimer's disease and cancer (Hinz and Brune, 2002; Dubois *et al.*, 1998), and the use of COX-2 deficient animals to investigate these events will be discussed later in this chapter (see **section 1.19**).

1.10 COX-3

In 2002 a so-called third COX isoform was described (Chandrasekharan *et al.*, 2002) and controversially named COX-3 despite not being a product of a separate gene. COX-3, along with two new smaller forms of COX-1 also described in this paper, are formed from the COX-1 gene by 'alternative splicing' of COX-1 mRNA. This results in a protein which is similar to COX-1 with the addition of an N-terminal extension due to the retention of intron-1 and the retained signal peptide. It was suggested that the

retention of intron 1 may change the way the enzyme molecule is folded, may affect dimerisation, or the conformation of the active site (Chandrasekharan *et al.*, 2002).

COX-3 was shown to be preferentially inhibited by acetaminophen (paracetamol) and may help to explain some of the unanswered questions as to the mode of action of acetaminophen. Often classified as an NSAID (see **section 1.18**), acetaminophen relieves pain and reduces fever, yet is not an effective anti-inflammatory medicine and does not markedly inhibit either COX-1 or COX-2. Chandrasekharan *et al.* demonstrated that COX-3 is strongly expressed in the canine brain and may be involved in mediating pain, however this still leaves unanswered questions as the pyretic response has been associated with rapid induction of COX-2, not COX-1 (and therefore not COX-3, as a COX-1 gene product). Pyresis has been shown to be inhibited in mice deficient in COX-2, but not COX-1/3, (Li *et al.*, 1999) and COX-2 selective inhibitors are as good as traditional NSAIDs at reducing fever but these only react weakly with the COX-1/3 enzymatic site (Luong *et al.*, 1996; Kurumbail *et al.*, 1996; Riendeau *et al.*, 1997). Previous data has also indicated a COX-2 variant involved in pain. The same group to describe COX-3 have also previously suggested an existence of an isoform of COX-2 that is particularly sensitive to acetaminophen (Simmons *et al.*, 1999; Botting *et al.*, 2000), and Willoughby *et al.* suggested the existence of an inducible COX-3 involved in the resolution of inflammation which unlike COX-1 and COX-2 does not produce pro-inflammatory prostanoids but anti-inflammatory members of the prostanoid family (Willoughby et al., 2000). Both Willoughby and Botting hypothesised that this new enzyme included the COX-2 specific carboxyl terminus which allowed it to be detected by COX-2 specific antibodies. Whether or not COX-3 and the COX-2 derived variants are true isoforms, or splice variants, it is interesting that multiple COX isoenzymes could be derived from just two distinct genes.

However, in a recent letter to the editor published in Immunology Letters, the true existence of COX-3 has been questioned by Dinchuk and colleagues. Chandrasekharan described that the length of intron 1 in human COX-3 differed by one nucleotide from the sequence observed in the dog and suggested that this may have been a sequencing error. However, Dinchuk raises the issue that if not a sequencing error, this would shift the remainder of the protein out of frame with respect to the open reading frame of COX-1. After cloning COX-1 cDNAs and human genomic fragments spanning the region, Dinchuk found these clones to agree with the published human COX-1 intron 1

sequence of 94, not 93 nucleotides as seen in the dog, and therefore suggest that none of the downstream alternative splices are able to rescue the reading frame of the proposed COX-3 in humans (Dinchuk *et al.*, 2003). This suggests that a full-length, catalytically active form of COX-3 does not appear in humans, however further research is required.

1.11 PGE synthases

The final stage in generation of specific prostaglandins from PGH_2 involves the terminal PG synthases. Specific synthases have different structures and exhibit cell- and tissue-specific distributions. Two synthases have been described for PGE_2 synthesis, namely cytosolic PGE_2 synthase (cPGES) and perinuclear membrane-bound PGES (mPGES, Beuckmann *et al.*, 2000). cPGES is constitutively expressed in a wide variety of cells and tissues and is associated with heat shock protein 90 (HSP90) whereas mPGES is a perinuclear protein belonging to the microsomal glutathione-S-transferase (GST) family. Its expression is strongly induced by pro-inflammatory stimuli and is also down regulated by anti-inflammatory glucocorticoids (Murakami *et al.*, 2000; Thoren and Jakobsson, 2000).

It has been hypothesised that there is selective functional linkage between COX isoforms and the PGES. This idea is supported by studies using co-expression and antisense which have shown that the constitutive cPGES and the inducible mPGES favour COX-1 and COX-2 respectively (Tanioka *et al.*, 2000; Murakami *et al.*, 2000). Similarly to the expression of the COX isoforms to which they are coupled, this linkage may aid in controlling the spatial and temporal production of PGE_2 in a tissue and stimulus-dependent fashion.

Recently, it has been shown that mice lacking inducible mPGES show impaired inflammatory and pain responses (Trebino *et al.*, 2003). These animals are viable and fertile and develop normally compared with wild type controls, however homozygote deficient animals display a marked reduction in inflammatory responses compared with wild type mice in multiple assays.

1.12 PGE receptors

There are at least 9 known prostaglandin receptors, and PGE_2 signals via four separate G-coupled rhodopsin-type receptors, each with seven transmembrane domains encoded for by a different gene, namely EP1-4 (Coleman *et al.*, 1994). Most EP receptors are localised at the plasma membrane although some are situated at the nuclear envelope (Bhattacharya *et al.*, 1998). The receptors also differ both in the cell type in which they are expressed, and the intracellular pathways which they signal through. Thus the existence of subtype receptors allows PGE_2 to have diverse and opposing actions in different cell types. EP2 and EP4 are considered 'relaxant' receptors and signal through G_s-mediated increases in intracellular cAMP. The 'contractile' receptor EP1 signal though G_q-mediated increases in intracellular calcium. And EP3 is regarded as an 'inhibitory' receptor that couples to G_i and decreases cAMP formation. In fibroblasts, PGE_2 has been shown to reduce α_1 (I) collagen expression via the EP2 receptor and consequent rise in cAMP (Choung *et al.*,1998) and PGE_2 inhibits the fibroblast to myofibroblast transition via EP2 signalling and cAMP elevation (Kolodsick *et al.*, 2003). Knockout mice for each receptor subtype have been generated (Ushikubi *et al.*, 1998) and these animals may help establish a role for the receptors in pulmonary disease in the future. A recent publication has also described the first use *in vivo* of an EP2 receptor-selective PGE_2 agonist. This agonist was administered locally into bones, and successfully induced bone repair in a fracture model in rats without the side effects of administering PGE_2 (Paralkar *et al.*, 2003, Grant *et al.*, 1991).

1.13 Inactivation of PGE_2

In addition to synthesis, degradation is a rapid mechanism by which physiological levels of prostaglandins can be altered. The liver and lungs are responsible for their inactivation (Piper *et al.*, 1970; Samuelsson., 1964) and over 90% of PGE_2 is inactivated during a single passage through the pulmonary vascular bed (Ferreira and Vane, 1967).

Inactivation of PGE_2 occurs intracellularly and first requires carrier-mediated transport across the cell membrane as prostaglandins diffuse poorly (Bito and Baroody, 1975; Schuster *et al.*, 1998). This is followed by intracellular catabolism (Ensor and Tai, 1995; Schuster *et al.*, 1998) of which the first reaction, enzymatic oxidation of the 15-

hydroxyl group, is crucial because it leads to the loss of biological activities (Ensor and Tai, 1995). This reaction is catalysed by 15-hydroxy-PG dehydrogenase (15-PGDH or PGDH) and less importantly, carbonyl reductase (CR). PGDH is found in many mammalian tissues and is known to be inducible and responsive to steroid hormones (Greenland *et al.,* 2000). NSAIDs also inhibit PGDH (Cho and Tai, 2002), and genetic deletion of PGDH leads to an increase in circulating blood/tissue levels of PGE_2 (Coggins *et al.,* 2002).

1.14 PGE_2 in the lung

Prostaglandins are formed by most cells in our bodies and act as both autocrine and paracrine lipid mediators. PGE_2 is the major prostanoid in the lung and has been reported to be present in bronchoalveolar lavage fluid at 20ng/ml, 80-fold higher than in plasma (0.25ng/ml, Ozaki *et al.,* 1987). However, this value may not represent a truly basal level as bronchoalveolar lavage itself may induce PGE_2 synthesis. It is synthesised by fibroblasts, epithelial cells, smooth muscle cells, endothelial cells and macrophages and is thought to have bronchoprotective effects. These include bronchodilation, inhibition of eosinophil chemotaxis and inhibiting release of mediators from mast cells in the lung (Pavord and Tattersfield, 1995).

Synthesis of PGE_2 by fibroblasts in the lung can be induced by a range of pro-fibrotic and pro-inflammatory mediators known to be increased in the pathogenesis of pulmonary fibrosis, such as TGF-β, IL-1β, TNF-α and PDGF (McAnulty *et al.,* 1995; 1997; Diaz *et al.,* 1989; Keerthisingam *et al.,* 2001; Wilborn *et al.,* 1995; Mauviel *et al.,* 1988; Habenicht *et al.,* 1985). TNF-α and IL-1β are also known to increase PGE_2 production via increasing the steady-state levels of COX-2 mRNA (Diaz *et al.,* 1992). PGE_2 is thought to have a negative feedback effect on PDGF production which counteracts its effect on fibroblasts proliferation (Boyle *et al.,* 1999) and is a potent inhibitor of TGF-β_1-induced fibroblast proliferation and collagen synthesis (McAnulty *et al.,*1995; 1997, see **section 1.4.4**). Inflammation also induces production of PGE_2 in the lung (see **section 1.16**). Using the bleomycin model of pulmonary fibrosis, increased levels of PGE_2 in plasma (Chandler and Giri, 1983) and lung homogenate (Giri and Witt, 1985) have been shown following injury in hamsters, and in bronchoalveolar lavage fluid in mice (Moore *et al.,* 2000). Furthermore, vanadium pentoxide (V_2O_5 , Bonner *et*

al., 2001) and ovalbumin (Gavett *et al.*, 1999) have also been shown to increase levels of PGE_2 in the lungs of mice.

In pulmonary fibrosis there may be failure in this tightly regulated mechanism, and defective suppression of pro-inflammatory and pro-fibrotic cytokines may lead to unrestrained fibroblast proliferation and collagen production in the fibroblastic foci. Mechanisms for a failure to upregulate PGE_2 synthesis in pulmonary fibrosis will be discussed in detail in **section 1.17**

1.15 Cellular effects of PGE_2

PGE_2 is known to inhibit an array of fibroblast functions known to be important in the pathogenesis of pulmonary fibrosis such as fibroblast proliferation (Elias *et al.* ,1988; Bitterman *et al.*, 1986), collagen synthesis (Korn *et al.*, 1980; Goldstein and Polgar, 1982; Saltzman *et al.*, 1982), fibroblast to myofibroblast differentiation (Kolodsick *et al.*, 2003), fibroblast chemotaxis (Kohyama *et al.*, 2001) and contraction of ECM matrices (Skold *et al.*, 1999; Zhu *et al.*, 2001). In addition it has also been shown to promote degradation of newly synthesised collagen (Baum *et al.*, 1980; Brilla *et al.*, 1995). The following section will discuss how PGE_2 is responsible for these cellular effects and discuss potential mechanisms for its action.

Addition of exogenous PGE_2 has been shown to down regulate α_1 (I) collagen gene expression in lung fibroblasts induced by TGF-β_1 (Fine *et al.*,1989) and TNF-α (Diaz *et al.*,1989), and attenuate collagen type I and III production induced by IL-1 (Goldring and Krane, 1987). It is known that PGE_2 raises intracellular cAMP levels, suggesting a potential causal relationship between levels of cAMP and modulation of collagen metabolism and fibroblast proliferation via the EP2 and/or EP4 receptors which act via stimulation of cAMP. Indeed, it has been shown that PGE_2 can reduce α_1 (I) collagen RNA expression via the EP2 receptor and consequent rise in cAMP (Choung *et al.*,1998) and previous work has suggested that elevating cAMP levels directly in IMR-90 cells suppresses collagen production (Polgar *et al.*, 1979; Clark *et al.*, 1982)

A simultaneous increase in levels of cAMP has also been demonstrated following the inhibition of collagen production and increased intracellular degradation of newly synthesised collagen by PGE_1 (Baum *et al.*, 1980; Barile *et al.*, 1988, Brilla *et al.*,

1995). Furthermore, this can be duplicated with cAMP agonists and inhibited with antagonists (Baum *et al.,* 1980). A role for cAMP in PGE_2-mediated inhibition of proliferation has been demonstrated in arterial smooth muscle cells and hepatic stellate cells (Bornfeldt *et al.,* 1997; Mallat *et al.,* 1998) and the cAMP analogue dibutryl cAMP has been shown to block the proliferation of human lung fibroblasts in response to serum (Fine and Goldstein, 1987). PGE_2-mediated inhibition of fibroblast chemotaxis has also been shown to act via a cAMP dependent mechanism where the inhibitory effect of PGE_2 was itself inhibited by a cAMP-dependent protein kinase (PKA) inhibitor suggesting that the cAMP dependent effect is mediated by PKA (Kohyama *et al.,* 2001). PGE_2 has also been shown to inhibit the expression of lysyl oxygenase, the enzyme which catalyses the first step in the cross-linking of collagen fibrils required for the deposition of collagen into the ECM. Again, this has been shown to be mediated via cAMP and EP2 receptors (Choung *et al.,* 1998).

The ability of PGE_2 to inhibit fibroblast to myofibroblast transition has also been shown to be mediated by EP2 signalling and subsequent increases in cAMP production. In a recent study, Kolodsick and colleagues showed that the inhibitory effects of PGE_2 on TGF-β-induced α-SMA expression in IMR-90 fibroblasts are mimicked by an EP2 selective agonist, butaprost, and forskolin-induced direct activation of adenyl cyclase. Conversely, an EP2 antagonist was shown to block the inhibitory effects of PGE_2 (Kolodsick *et al.,* 2003).

However, cAMP-independent mechanisms have also been shown to downregulate α_1 (I) collagen gene transcription, possibly acting via a PGE_2 inducible protein (Fine *et al.,* 1992). More recently PGE_2 has been shown to inhibit transcription of the CTGF gene which is itself induced by TGF-β and directly activates α_1 (I) collagen mRNA transcription (Leof *et al.,* 1986). The anti fibrotic effects of PGE_2 may therefore involve inhibition of CTGF transcription (Ricupero *et al.*, 1999). PGE_2 has also been shown to downregulate signalling TGF-β receptors which could limit collagen gene expression (Fine *et al.,*1995).

1.16 PGE_2 and inflammation

Aspirin and other non-steroidal antiinflammatory drugs (NSAIDs) are the most widely used class of drug world-wide (see **section 1.18**). Amongst other indications, they are

commonly used in the treatment of chronic inflammatory diseases and inhibit the production of prostanoids, including PGE_2, via inactivation of COX. As previously mentioned, prostaglandins (PGs) derived from COX-1 are often described as performing homeostatic functions such as gastric cytoprotection, whereas those derived from COX-2 are seen as pathogenic and associated with inflammation. For this reason COX-2 selective NSAIDs have been developed over the last 15 years with the aim of inhibiting COX-2-derived PGs whilst maintaining the production of 'housekeeping' levels of COX-1-derived PGs and therefore avoiding the side effects associated with NSAIDs. The use of COX-2 selective NSAIDs for rheumatoid arthritis is now widespread (Simon *et al.,* 1998). Pro-inflammatory mediators such as IL-1 or TNF-α increase production of PGE_2 via the induction of COX-2 (Endo *et al.,* 1995) and one mechanism of action of the powerful anti-inflammatory steroid dexamethasone is as an inhibitor of IL-1β-induced COX-2 at both the transcriptional and post-transcriptional level. Therefore, there is considerable evidence for the role of PGE_2 in both promoting and modulating inflammation.

In vivo, administration of PGE_2 appears to promote inflammation by increasing vascular permeability and potentiating the effects of pro-inflammatory mediators (Williams *et al.*, 1979; Agro *et al.,* 1996; Serhan *et al.,* 2000). *In vivo* administration of PGE_2 neutralising antibodies have also been shown to reduce paw oedema, hyper-analgesia and IL-6 production following carrageenan and adjuvant-induced inflammation (Portanova *et al.,* 1996). In the lung, administration of NSAIDs have been used to examine the role of the inflammatory response in the bleomycin model of pulmonary fibrosis. The first such study was that of Thrall and co-workers who showed that treatment with indomethacin ameliorates bleomycin-induced fibrosis in rats. After 60 days, treatment with indomethacin resulted in reduced lung collagen accumulation compared with bleomycin alone, although this was not seen at day 14 and no mention is made of intermediate timepoints. Histopathologic evidence of pulmonary fibrosis was reduced following indomethacin treatment, as was the reported bleomycin-induced eosinophilia (Thrall *et al.,* 1979). Following this, diclofenac acid (Voltaren) was used in a hamster model where pre-treatment before intratracheal bleomycin administration followed by daily injections of the NSAID reduced lung collagen accumulation at both 14 and 21 days compared with bleomycin alone. However, no assessment of PGE_2 levels were made in this study (Chandler and Young, 1989). Indomethacin was again used by Mall and co workers following systemic administration of bleomycin in rats.

Histological analysis revealed reduced fibrosing alveolitis in the bleomycin-treated animals and quantitative morphometric analysis indicated that treatment with indomethacin reduced alveolar wall thickening, fibrosis and inflammatory cell accumulation. However, animals treated with indomethacin lost over 50% of their body weight throughout the experiment and had reduced lung volumes which was not seen in control animals which received bleomycin alone (Mall *et al.*, 1991).

However, these three studies all used continuous administration of the NSAID from the time of bleomycin instillation. The bleomycin model is characterised by an acute inflammatory response in the first 7 days prior to the increase in total lung collagen accumulation typically seen by day 14. In a slightly different approach, Moore and co-workers delayed administration of indomethacin until 10 days after bleomycin instillation when the acute inflammatory response has decreased. In this experiment contrasting results were observed; animals which received indomethacin showed an enhanced fibrotic response compared with control mice suggesting that PGE_2 is protective against bleomycin-induced lung injury (Moore *et al.*, 2000).

The cellular effects of PGE_2 have also been shown to be anti-inflammatory. Stimulated macrophages and neutrophils both produce PGE_2 via COX-2 (Brook *et al.*, 1999; Maloney *et al.*, 1998) and its effects on these cells are inhibitory as PGE_2 promotes macrophage deactivation (Strassman *et al.*, 1994), and reduces macrophage (Zeidler *et al.*, 2000) and neutrophil chemotaxis and aggregation (Armstrong *et al.*, 1995; Wise *et al.*, 1996). In fibroblasts, although PGE_2 is induced by the pro-inflammatory cytokines TGF-β_1, IL-1β, TNF-α and PDGF (Diaz *et al.*, 1989; McAnulty *et al.*, 1995; 1997; Yucel-Lindberg *et al.*, 1995; Mauviel *et al.*, 1988; Habenicht *et al.*, 1985) it has also been shown to form a negative feedback loop on these cytokines. This is demonstrated for TGF-β_1 and PDGF on fibroblast proliferation (McAnulty *et al.*, 1997; Boyle *et al.*, 1995) and TGF-β_1 on collagen production (McAnulty *et al.*, 1995). PGE_2 has also been shown to stimulate the production of anti-inflammatory cytokines such as IL-10 (Strassman *et al.*, 1994) and inhibits the production of pro-inflammatory cytokines from macrophages (Fadok *et al.*, 1998; Standiford *et al.*, 1992) as well as reducing respiratory burst and degranulation in neutrophils (Fantone *et al.*, 1984; Weksler and Goldstein, 1980). Synthesis of pro-inflammatory leukotrienes (Christman *et al.*, 1993) and IgE (Pene *et al.*, 1988) are also inhibited by PGE_2 and products of COX-2 also produce anti-

inflammatory mediators such as PPAR-γ (Gilroy *et al.,* 1999) and lipoxins (Levy *et al.,* 2001).

When given alone *in vivo*, PGE_2 does not produce significant levels of pain, swelling or neutrophil infiltration (Portanova *et al.,* 1996; Serhan *et al.,* 2000). Specifically in the lung, PGE_2 is bronchodilatory (Mathe and Hedqvist, 1975) and administration of PGE_2 blocks both the early and late response to antigen challenge in asthmatics (Pavord *et al.,* 1993). PGE_2 administration also prevents airway hyper-reactivity in aspirin-intolerant asthmatics (Sladek *et al.,* 1994, Sestini *et al.,* 1996). A study by Grant and co-workers showed that continuous intravenous infusion of PGE_1 for 22 hours in a hamster model of bleomycin-induced pulmonary fibrosis lead to decreased neutrophil influx and plasma leak in the lungs of treated animals (Grant *et al.,* 1991). This suggests a protective role for PGE_2 in the inflammatory response and both COX-1 and COX-2 deficient mice showed increased inflammatory response following antigen challenge (see **section 1.19**, Gavett *et al.,* 1999), and COX-2 deficient mice show an increased inflammatory response to vanadium pentoxide challenge in the lung compared to both wild type and COX-1 deficient mice (Bonner *et al.,* 2002). However, as shown in **section 1.19**, not all studies using COX-2 deficient mice conclude that the inflammatory response is enhanced in these animals. Together these data suggest that the role for PGE_2 and other products of the COX pathway in the inflammatory response is complex.

1.17 Reduced expression of PGE_2 and COX-2 in pulmonary fibrosis

Despite the increased expression of TGF-β and other pro-fibrotic mediators which induce its synthesis, levels of PGE_2 in BALF from patients with IPF have been shown to be 50% lower than in normal individuals (Borok *et al.,* 1991). Macrophages obtained from patients with IPF also show a reduced capacity to produce PGE_2 (Ozaki *et al.*,1987). Furthermore, fibroblasts derived from the lungs of patients with IPF show both decreased basal levels of synthesis of PGE_2 compared with normal cells (Wilborn *et al.,* 1995; Keerthisingam *et al.,* 2001, Marchand-Adam *et al.,* 2003), and fail to induce PGE_2 synthesis on stimulation with IL-1β, TNF-α, LPS or TGF-$β_1$ (Wilborn *et al.,* 1995; Keerthisingam *et al.,* 2001; Vancheri *et al.,* 2000). However, this is contradicted by Cruz-Gervais and colleagues who have shown PGE_2 levels to be unaltered in fibrotic fibroblasts compared with control cells. Despite this report, the

majority of data suggest that PGE_2 deficiency is associated with several cell types in fibrotic lung disease.

Using fibroblasts derived from patients with IPF it has been shown that the failure to induce synthesis of PGE_2 in response to an array of pro-fibrotic mediators is associated with a decreased capacity to upregulate COX-2 (Wilborn *et al.*, 1995; Keerthisingam *et al.*, 2001). Wilborn demonstrated that fibroblasts derived from the lungs of patients with pulmonary fibrosis express normal levels of COX-1 protein and radiolabelling studies showed these fibroblasts to be able to release endogenous AA, excluding a defect in the $cPLA_2$-mediated release of AA in these cells. Furthermore PGE_2 was also synthesised when the endoperoxidase intermediate PGH_2 was added to the fibrotic cells, bypassing the COX pathway, excluding a defect in PGES. However, COX-2 protein expression was significantly decreased in fibrotic lung fibroblasts (Wilborn *et al.*, 1995), and this laboratory has also shown this to be true at the mRNA level (Keerthisingam *et al.*, 2001). This failure to upregulate COX-2 leads to a more fibrogenic phenotype in these cells characterised by a loss in the antiproliferative response to TGF-β_1 and an increase in procollagen production following stimulation with TGF-β_1 (Keerthisingam *et al.*, 2001).

Reasons for the failure to upregulate COX-2, and subsequently synthesis of PGE_2 in pulmonary fibrosis are currently unknown. However, there may be several potential explanations. Functional variants in the COX-2 gene may affect gene expression or mRNA stability leading to a loss in inducibility. Recent work from this laboratory has described a novel functional change in the 5' transcriptional regulatory promoter region of the COX-2 gene (-765G>C). This polymorphism has been shown to affect the transcription rate of a reporter gene in transient transfection studies (Papafili *et al.*, 2002). Future work may determine the role of this polymorphism in fibrotic lung disease. Alternatively, since there is no evidence to suggest that fibroblasts derived from patients with pulmonary fibrosis show a general defect in their response to pro-fibrotic mediators, other than a failure to induce COX-2, there may be dysregulation of the transcription factors or signalling molecules upstream of the COX-2 gene. Finally, the inability to induce COX-2 could reflect an acquired defect that is specific to the lung. For example, viral infection could alter a cells ability to induce COX-2. EBV, a herpesvirus previously associated with the development of pulmonary fibrosis (Egan *et al.*, 1997; Tang *et al.*, 2003), has been shown to inhibit COX-2 expression both at the

transcriptional and translational levels, resulting in suppression of PGE_2 biosynthesis in monocytes (Savard *et al.*, 2000).

Together, these data suggest that a decreased capacity to upregulate synthesis of PGE_2 via dysregulation of COX-2 expression may play a key role in the pathogenesis of pulmonary fibrosis. This thesis aims to address this hypothesis *in vivo* by inhibiting the function of COX-2, and therefore presumably PGE_2 synthesis, both pharmacologically and using COX-2 deficient mice, in the bleomycin model of lung fibrosis. The final sections in this chapter discuss pharmacological inhibition of COX-2 and COX-2 gene disrupted animals.

1.18 Pharmacological inhibition of COX

Non-steroidal anti-inflammatory drugs (NSAIDs) have anti-inflammatory, analgesic, and anti-pyretic effects. In 1971, Vane showed the action of NSAIDs to be due to the inhibition of cyclooxygenase and production of pro-inflammatory prostaglandins (Vane, 1971). All classical NSAIDs compete with arachidonate for binding to the cyclooxygenase active site and therefore inhibit both COX-1 and COX-2, but in general preferentially inhibit COX-1 (Munroe and Lau 1995; DeWitt, 1999). NSAIDs are competitive inhibitors which interfere with fatty acid substrate binding in one of three general modes of inhibition (Munroe and Lau, 1995; DeWitt, 1999): either rapid, reversible binding (e.g. ibuprofen); or rapid, lower affinity reversible binding followed by time-dependent, higher affinity slowly reversible binding (e.g. flurbiprofen); or finally, rapid, reversible binding followed by covalent modification, as in the acetylation of Ser530 by aspirin.

The use of NSAIDs is associated with problematic side effects including gastrointestinal toxicity where they have been shown to induce ulceration and promote bleeding of existing ulcers. Renal toxicity has also been associated with the use of NSAIDs, and the use of COX inhibitors in aspirin-intolerant asthmatics causes adverse respiratory reactions (reviewed by Szczeklik and Stevenson, 1999). With the identification of COX-2, and its induction by inflammatory agents, it has been proposed that the anti-inflammatory effects of NSAIDs are due to inhibition of COX-2, whereas the side effects including gastrointestinal and renal toxicity are due to inhibition of the 'housekeeping' actions of COX-1. The structural differences between the COX sites of

the two isoforms have permitted the development of a new generation of isoform selective inhibitors (Luong *et al.,* 1996) designed to exhibit many-fold higher selectivity towards COX-2 (Munroe and Lau, 1995; DeWitt, 1999) such as celecoxib, rofecoxib and NS398. Celecoxib has been licensed in the treatment of patients with osteoarthritis and rheumatoid arthritis, and rofecoxib for the treatment of patients with osteoarthritis and acute musculoskeletal pain and are largely free of gastrointestinal side effects. However, in other reports, both ulceration and GI toxicity have been linked to the use of selective COX-2 inhibitors (Reuter *et al.,* 1996) suggesting that COX-2 derived metabolites play a role in maintaining the integrity of the gastric mucosa. This data is also supported by *in vivo* work by Morteau who showed that COX-$2^{-/-}$ mice show enhanced experimentally-induced colitis compared with WT animals (Morteau *et al.,* 2000).

NS398 is one of the most selective COX-2 inhibitors (Futaki *et al.,* 1993; Futaki *et al.,*1994; Warner *et al.,* 1999), and although not used clinically, has been studied extensively *in vivo.* NS398 has previously been shown to be highly selective for COX-2 and to significantly inhibit inflammation-induced PGE_2 production *in vivo* at doses as low as 1-1.5mg/kg (Masferrer *et al.,* 1994; Payá *et al.,* 1997; Futaki et al., 1997) without affecting constitutive PG synthesis in the stomach (Masferrer *et al.,* 1994). At 1 and 10mg/kg NS398 has previously been shown not to inhibit COX-1 activity in mouse granuloma tissue (Gilroy *et al.,* 1998), and at doses of up to 30mg/kg body weight it has been shown not to inhibit COX-1 activity either systemically or in the stomach of mice (Wallace *et al.,* 1998). This thesis has used NS398 to investigate the relative contribution of COX-1 and COX-2 in PGE_2 synthesis following bleomycin-induced lung injury and a dose of 3mg/kg administered orally twice daily was used to maximise inhibitory effect on COX-2, whilst maintaining selectivity by not inhibiting COX-1 activity.

1.19 COX-2 deficient mice

Transgenic mice have proved useful tools in identifying the role of specific mediators in disease and therefore COX-2 deficient (COX-$2^{-/-}$) mice appear to be an ideal model to investigate the effect of COX-2 deficiency on the development of experimentally induced pulmonary fibrosis. To date, COX-$2^{-/-}$ mice have provided valuable information concerning some of the functions of COX-2 both in physiological processes and

disease. Two strains of COX-2 deficient mice have been generated; Morham and colleagues disrupted exon 8 (termed COX-$2^{ex8/ex8}$) of the COX-2 gene whilst Dinchuk et al disrupted exon 1 (termed COX-$2^{ex1/ex1}$, Morham *et al.*, 1995; Dinchuk *et al.*, 1995). Phenotypic characteristics of COX-$2^{-/-}$ mice are summarised in **Table 1.3**.

Organ/process	Effect of COX-$2^{-/-}$ phenotype	References
Viability	Not lethal in utero Reduced neonatal viability	Dinchuk *et al.*, 1995; Morham *et al.*, 1995
Fertility	Reduced fertility and null females infertile. Effects on ovulation, fertilisation, implantation and decidualisation	Dinchuk *et al.*, 1995; Morham *et al.*, 1995; Lim *et al.*, 1997; Davis *et al.*, 1999
Prostaglandin synthesis	Normal constitutive synthesis Decreased inducible synthesis	Dinchuk *et al.*, 1995; Morham *et al.*, 1995; Morteau *et al.*, 2000; Bonner *et al.*, 2002
GI tract	No spontaneous gastric ulceration or inflammation Increased DSS-induced colitis	Dinchuk *et al.*, 1995; Morham *et al.*, 1995; Morteau *et al.*, 2000
Kidney	Renal dysplasia, impaired postnatal renal development	Dinchuk *et al.*, 1995; Morham *et al.*, 1995
Lung	Increased allergic responses and inflammation to ovalbumin sensitisation and challenge No difference in LPS-induced inflammation Enhanced inflammatory and fibrotic response to V_2O_5 Decreased severity of cerulein-induced pancreatitis-associated lung injury	Gavett *et al.*, 1999; Zeldin *et al.*, 2001 Bonner *et al.*, 2002 Song *et al.*, 2002; Etheridge *et al.*, 2002;
Carcinogenesis	Decreased tumour development	Oshima *et al.*, 1996; Chulada *et al.*, 2000; Zhang *et al.*, 2000; Tiano *et al*, 2002
Pancreas	Decreased severity of cerulein-induced pancreatitis	Song *et al.*, 2002; Etheridge *et al.*, 2002
Heart	Cardiac fibrosis Increased cardiac ischemia/reperfusion injury	Dinchuk *at al.*, 1995; Camitta *et al.*, 2001
Peritoneum	Peritonitis and multiple adhesions	Dinchuk *et al.*, 1995
Bone	Normal skeletal development Decreased bone resorption Decreased fracture healing Decreased autoimmune arthritis	Okada *et al.*, 2000; Zhang *et al.*, 2001; Zhang *et al.*, 2002; Myers *et al.*,,2000
Brain	Decreased febrile response Decreased ischemic brain injury	Li *et al.*, 1999; Li *et al.*, 2001; Iadecola *et al.*, 2001

Table 1.3 Phenotypic characteristics of COX-$2^{-/-}$ mice. Modified from Loftin *et al.*, 2002

Female COX-2 homozygote mice are infertile and both sexes show reduced viability. This increased mortality has been shown to be due to chronic renal failure secondary to renal developmental abnormalities, including fibrosis. Fibrotic abnormalities of the heart and ovaries have also been described and an increased incidence of peritoneal adhesions (Dinchuk *et al.,* 1995; Morham *et al.*, 1995).

These mice have been used to investigate the role of COX-2 pathological conditions, including cancer, where COX-2 deficiency has been shown to protect against tumourgenesis (Chulada *et al.,* 2000; Zhang *et al.,* 2000 Oshima *et al.,* 1996; Tiano *et al,* 2002). Neither Morham or Dinchuk described alterations in inflammatory responses to either tetradecanoyl phorbol acetate (TPA) or arachidonic acid (Dinchuk *et al.*,1995; Morham *et al.*, 1995; reviewed by Langenbach *et al.,* 1999). However COX-2 deficient mice do show an enhanced colonic inflammation following DSS-induced colitis (Morteau *et al.*,2000). In the lung, it has been shown that COX-2 deficient mice show an enhanced allergic response following ovalbumin challenge with enhanced pulmonary eosinophilia and lymphocytosis (Gavett *et al.*, 1999) although following administration of LPS there is no enhanced inflammatory response. Recently Bonner and colleagues described an enhanced inflammatory response to the transition metal V_2O_5 . This was accompanied by an increase in lavage TNF-α levels and an increase in lung collagen production suggesting that COX-2 may be protective in fibrotic disease (Bonner *et al.,* 2002). This agrees with previous histological data from our laboratory showing that COX-2 deficient mice exhibit an enhanced fibroproliferative response to bleomycin (Keerthisingam *et al.,* 2001). Despite our preliminary work, and that of Bonner, the response of COX-$2^{-/-}$ mice to fibrotic stimuli such as bleomycin remains to be fully evaluated and the data presented in this thesis provides additional insight into the role of COX-2 in both the inflammatory and fibrotic processes.

1.20 Summary and aims of study

Pulmonary fibrosis is a chronic and progressive process leading to severe respiratory insufficiency and ultimately death of those affected individuals. Prognosis is poor and current treatment is inadequate. The pathogenesis of pulmonary fibrosis is incompletely understood, but from what is known it is clear that two processes appear to be central in its development and progression; namely the inflammatory response to injury, followed by the deposition of extracellular matrix proteins, particularly collagens in the lung.

Many of the cellular and molecular pathways involved in mediating these two processes are tightly regulated by soluble mediators such as cytokines, growth factors and lipid-mediators and it is widely accepted that the dysregulation of these mediators is an important underlying molecular mechanism in the pathogenesis of pulmonary fibrosis.

Once such mediator is prostaglandin E_2 (PGE_2) which is a potent inhibitor of fibroblast proliferation, collagen production, chemotaxis and myofibroblast differentiation in the lung. Despite the increased expression of pro-fibrotic mediators such as TGF-β known to induce its synthesis, levels of PGE_2 in both BALF and lung fibroblasts from patients with pulmonary fibrosis have been shown to be reduced compared with controls. *In vitro*, this failure to upregulate PGE_2 biosynthesis has been associated with a decreased capacity to upregulate the inducible isoform of the key enzyme in its biosynthesis, cyclooxygenase-2 (COX-2). This results in a more fibrotic phenotype in these cells, characterised by a loss in the anti-proliferative response to TGF-β and increased procollagen production. Preliminary results have also shown that COX-$2^{-/-}$ mice show an enhanced fibroproliferative response to bleomycin-induced lung injury compared with WT animals.

Despite large amounts of indirect evidence that limited expression of COX-2 and PGE_2 contributes to the pathogenesis of pulmonary fibrosis there is no direct proof. Therefore this thesis will address the hypothesis that;

A failure to upregulate COX-2 and therefore PGE_2 biosynthesis will potentiate bleomycin-induced pulmonary fibrosis.

The specific aims of this thesis are:

- **To investigate the production of COX-1, COX-2 and PGE_2 in the lungs of wild type mice following bleomycin-induced injury.** The specific COX isoform(s) upregulated following bleomycin injury is(are) not known. This will be examined using western analysis. The *in vivo* pharmacological inhibition of COX-2 using NS398 will be used to investigate the production of PGE_2 in the lungs of wild type mice.

- **To investigate the fibrotic and inflammatory responses to bleomycin-induced lung injury in wild type mice following pharmacological inhibition of COX-2.** NS398 will be orally administered and the fibrotic response will be evaluated histologically, and biochemically by measuring total lung collagen production. The inflammatory response will be evaluated by counting total cell number in bronchoalveolar lavage fluid and differential cell counts.

- **To investigate PGE_2 production following bleomycin-induced lung injury in the absence of COX-2 using COX-2 deficient ($COX\text{-}2^{-/-}$ and $COX\text{-}2^{+/-}$) mice .** The response of these animals will be compared to those of wild type control mice.

- **To investigate the fibrotic and inflammatory responses to bleomycin-induced lung injury in COX-2 deficient mice.** The response of these animals will be compared to those of wild type control mice.

CHAPTER TWO:

MATERIALS AND METHODS

Section 1 *In vivo* inhibition of COX-2 function

The aim of this thesis is to investigate a possible protective role for COX-2 and PGE_2 in the bleomycin model of lung injury. This will be done by inhibiting COX-2 function *in vivo* in two ways; firstly, pharmacologically using the highly selective COX-2 inhibitor N-[-2-cyclohexyloxy]-4-nitrophenyl methanesulphonamide (NS398), and secondly, genetically, using COX-2 gene deficient mice ($COX\text{-}2^{-/-}$, $COX\text{-}2^{+/-}$). **Sections 2.1** and **2.2** describe animals used in this study, including COX-2 deficient mice, and **section 2.3** describes the pharmacological inhibition of COX-2 function.

2.1 Animals

COX-2 deficient ($COX\text{-}2^{-/-}$) and strain matched C57BL6/SV129 wild type ($COX\text{-}2^{+/+}$ Jackson Laboratory, Maine, USA) mice (stock numbers 101045 and 002476) were bred at University College London (UCL). F3 generation mice were mated and F4 generation mice were used as experimental subjects. Due to the infertility of female $COX\text{-}2^{-/-}$ mice (Dinchuk *et al.*, 1995; Morham *et al.*, 1995), COX-2 heterozygous ($COX\text{-}2^{+/-}$) females were bred with both $COX\text{-}2^{-/-}$ and $COX\text{-}2^{+/-}$ males to maximise the breeding colony. Litters were weaned at four weeks of age and subsequently genotyped using genomic DNA. The animals were housed at UCL Central Biological Services facility on a 12 hour light/12 hour dark cycle at 25°C. Food and water were available ad libitum.

2.1.1 Wild type mice

The breeding strategy also produced $COX\text{-}2^{+/+}$ littermate animals. The two strains of wild type mice each represent valid controls and have both been used in previously published work with COX-2 deficient mice (Keerthisingham *et al.*, 2001; Zeldin *et al.*, 2001). The majority of the work discussed in this thesis used wildtype littermate animals (coded B196), however the study using the COX-2 pharmacological inhibitor NS398 used only strain-mated control animals (coded B197). The strain of wild type mice used in particular experiments will be indicated on each figure.

2.2 Genotyping COX-2 deficient mice

$COX\text{-}2^{-/-}$ and $COX\text{-}2^{+/-}$ mice show no phenotypic differences in their appearances compared with each other, or littermate wild type animals and therefore each individual animal must be genotyped. This was done using polymerase chain reaction (PCR)

amplification of genomic DNA isolated from the tail, which involves amplifying a region of the COX-2 gene altered in transgenic mice. This method was previously established in our laboratory (Keerthisingam, 2000).

2.2.1 Extraction of genomic DNA

Ear punches were used to identify individual mice. Approximately 0.5cm of the tip of the mouse tail was removed using clean scissors and placed in a centrifuge tube together with a drop of blood from the cut surface of the tail. This was immediately snap frozen in liquid nitrogen and silver nitrate was applied to the tail wound to stop further bleeding. Frozen tail samples were thawed on a glass microscope slide kept on ice. The tissue was cut into approximately 1mm pieces using a clean scalpel and then sheared further. The pieces were transferred into a clean, autoclaved 1.5ml tube and the blood washed out of the original tube using 500µl of lysis buffer (0.1M EDTA, 0.9% w/v NaCl, 0.05M Tris, 1% w/v SDS) and transferred into the clean tube. After each sample the forceps and scalpel were washed with 70% ethanol to avoid contamination between samples. Proteinase K (BDH/Merck) was added to give a final concentration of 1µg/ml and the lid of the tube was wrapped in parafilm and inverted several times. Two tubes were placed in a 50ml centrifuge tube containing 25ml H_20 and placed in a shaking waterbath overnight at 37°C.

Following digestion the tubes were centrifuged (12000 x g, 5 min, RT) to pellet the remaining tissue. The supernatant was transferred to a clean, autoclaved 1.5ml centrifuge tube and the tissue pellet washed with 500µl lysis buffer, inverted several times and recentrifuged. The supernatant from this step was then added to the original supernatant. An equal volume of isopropanol was added and the tube inverted several times until a white precipitate of DNA was seen. The precipitate was pelleted by centrifugation (12000 x g, 5 mins, RT) and the supernatant decanted and discarded. Finally the pellet was washed with 1ml 70% (v/v) ethanol and inverted several times before being recentrifuged. The ethanol was decanted and the DNA pellet air dried before being dissolved in 100µl double-deionised water (ddH_20) overnight at room temperature.

2.2.2 DNA concentration

A 5µl aliquot of genomic DNA was diluted with 995µl distilled water in a 1.5ml centrifuge tube giving a dilution factor of 200. The purity and concentration of the DNA

was determined by spectrophotometry measuring the optical density (OD) at both 260 and 280nm. The purity was assessed by the OD_{260}:OD_{280} ratio, and a ratio of 1.7:1 indicated relatively pure DNA. DNA was quantified by measuring the OD_{260} and calculated using the formula:

DNA (μg/ml) = OD_{260} x 50 x dilution factor (200)

The samples of genomic DNA were diluted in ddH_20 to give working concentrations of 100μg/ml. If problems with protein contamination were encountered with the PCR, these stocks were further diluted to 10μg/ml. Both working stocks and genomic DNA were stored at 4°C.

2.2.3 PCR protocol

The COX-2 deficient mice were generated by replacing a 1.8Kb fragment of the gene containing exon 1 with a 1.6Kb neomycin cassette (Dinchuk *et al.,* 1995). The PCR amplifies a 922bp product from the endogenous COX-2 gene and a 1.4Kb product from the disrupted allele (**Figure 2.1**). This protocol was adapted from conditions suggested by the Jackson Laboratory and further modified within our laboratory (Keerthisingham, 2000).

Three primers (Gibco Life Sciences) were used to amplify the specific region; a common downstream primer (547), a specific upstream primer for the wild type allele (546) and a specific primer for COX-2 deficient alleles (013). The nucleotide sequences are shown in **Table 2.1** below:

Primer	Nucleotide sequence
546	5'-ATC TCA GCA CTG CAT CCT GC-3'
547	5'-CAC CAT AGA ATC CAG TCC GG-3'
013	5'-CTT GGG TGG AGA GGC TAT TC-3'

Table 2.1 Primer nucleotide sequences used in PCR to genotype COX-$2^{-/-}$ mice

2.2.4 PCR Conditions

Each well of the PCR microtitre plate (Hybaid, Middlesex, UK) contained 200ng DNA (2μl of a 100ng/μl solution) and 3μl distilled water overlayed with a drop of mineral oil.

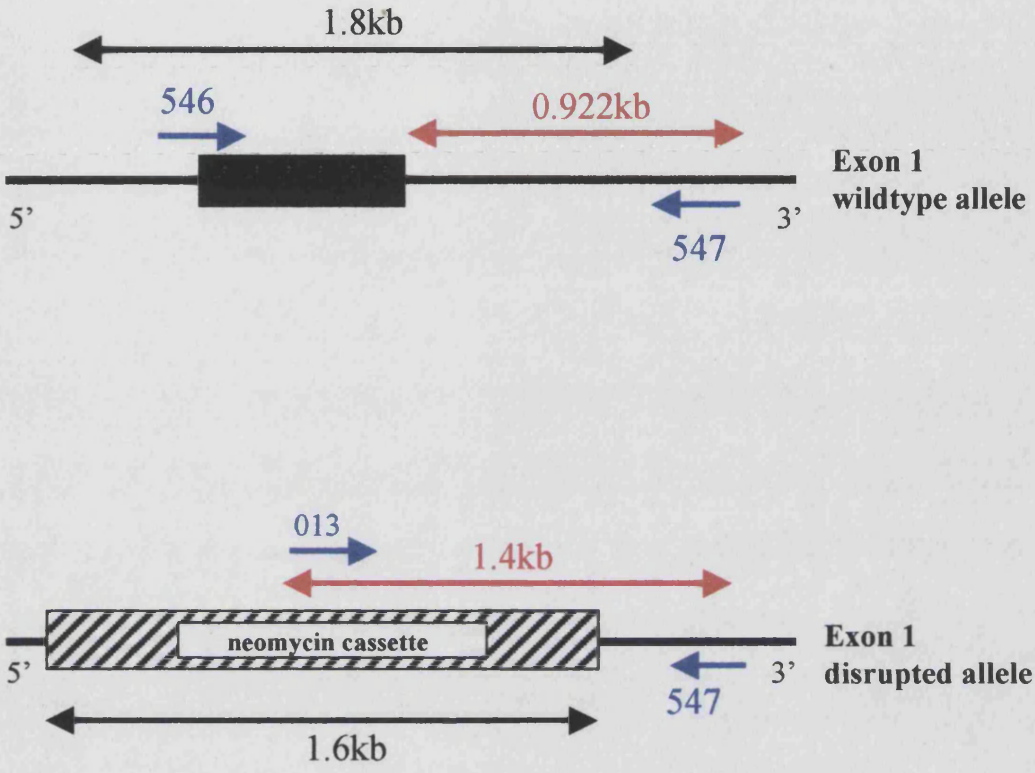
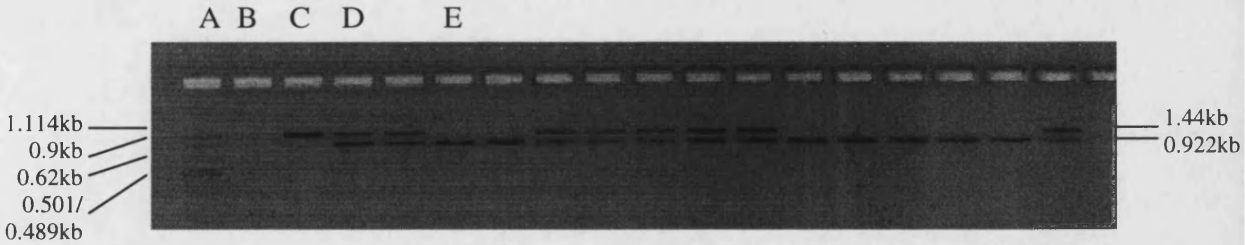


Figure 2.1 Disruption of exon 1 of the COX-2 gene in COX-2$^{-/-}$ mice
A 1.8Kb fragment of the gene containing exon 1 was replaced in the transgenic mice with a 1.6Kb neomycin cassette. The PCR primers 546 and 547 amplifies a 922bp product from the endogenous COX-2 gene and primers 013 and 547 amplified a 1.4Kb product from the disrupted allele.

Controls were used in each PCR using mouse DNA from confirmed genotypes and 5µl distilled water as a negative control. Sufficient reaction mixture to give a 10µl reaction volume (final concentrations 50mM KCL, 10mM dNTPs, 1.5mM $MgCl_2$, 0.2µM primer 546, 0.2µM primer 547 , 0.04µM primer 013) was prepared in a 1.5ml centrifuge tube. Taq DNA polymerase (5U/µl, Amersham Biosciences, Amersham, UK) was then added to the reaction mixture to give a final concentration of 2U/µl. After mixing, 5µl reaction mixture was added to each well under the mineral oil. The plate was then pulse centrifuged for 30secs (300 x g) to remove air bubbles and ensure total mixing of the components before being placed in a pre-programmed thermocycler (Peitler Thermocycler, MJ Research). The PCR conditions used were one cycle of initial denaturing (94°C, 3 mins), followed by 34 cycles of denaturing, annealing and extension (94°C, 1 min; 62°C, 2 mins; 72°C, 2 mins), with one cycle of final extension (72°C, 5 mins).

After the reaction was complete the products were separated by electrophoresis. A 2% (w/v) agarose (Ameresco, Ohio, USA) gel was prepared dissolved in 1x TBE buffer (0.09M Tris-borate, 0.002M EDTA, pH8). The gel solution was heated in a microwave for approximately 2 minutes at full power and then cooled under running water. Ethidium bromide was added to the gel at a final concentration of 0.5µg/ml. The gel was poured into a cast, 20 well combs placed in position and allowed to solidify at RT. Once solidified, the gel was transferred to the gel tank filled with 1x TBE running buffer and the combs removed. 5µl of 5x loading buffer was added to each well of the microtitre plate and 10µl of each product was run per well. A DNA molecular weight marker (Marker VIII, 0.25µg/ml, Boehringer Mannheim,) was run on each gel and the gel run for 25 minutes at 150 volts. The DNA bands were visualised using a phosphorimager (Fuji, FLA-3000) and the animal genotype determined by the band pattern. **Figure 2.2** shows an example of a PCR gel.



A Molecular weight marker
B Negative control
C COX-$2^{-/-}$ control
D COX-$2^{+/-}$ control
E COX-$2^{+/+}$ animal

Figure 2.2 Representative PCR gel

2.3 Pharmacological inhibition of COX-2

The highly selective COX-2 inhibitor NS398 (Cayman Chemicals/Alexis Corporation, Nottingham, UK) was dissolved in dimethylsulfoxide (DMSO, Sigma, UK) at 25mg/ml and stored as aliquots, at –40°C. Immediately prior to administration NS398 was thawed to room temperature, diluted in 1% Tween80 (Sigma, UK) phosphate buffered saline (PBS) to a concentration of 750µg/ml and administered to mice within one hour of preparation. Each animal received 3µg NS398/kg body weight in a final volume of 30µl, a dose known to selectively inhibit COX-2 without affecting COX-1 activity (see **sections 1.18, 4.3.1**). The inhibitor was administered by oral gavage every twelve hours as previously described (Gilroy *et al.*, 1998). Control animals received 30µl of vehicle alone. The first dose of NS398 was administered one hour prior to intratracheal instillation of bleomycin. To determine the effectiveness of NS398, PGE_2 was measured in bronchoalveolar lavage fluid (BALF) from both treated and control animals as described in **sections 2.5** and **2.7**.

Section 2 The bleomycin model of lung fibrosis

Animal models of pulmonary fibrosis have been useful tools for studying the pathogenesis of lung fibrosis. Bleomycin is an anti-neoplastic agent isolated from a strain of *Streptomyces verticillus* which exhibits dose-dependent pulmonary toxicity leading to progressive pulmonary fibrosis in 3-40% of patients treated with the drug. However, the toxic effects have been utilised advantageously in animal models where bleomycin-induced lung injury resembles biochemical and functional changes

associated with human pulmonary fibrosis with the exception that it is non-progressive (Schrier *et al.,* 1983; Clark *et al.,* 1983; Thrall and Barton, 1984; Aso *et al.,* 1976; Borzone *et al.,* 2001).

Bleomycin has been administered intravenously, intramuscularly, intraperitoneally, and intratracheally (IT) and all lead to an acute inflammatory response in the first 7 days which precedes matrix deposition and fibrosis characterised by an increase in lung collagen content 14-21 days following instillation of bleomycin. The inflammatory response to bleomycin instillation varies dependent on route of administration and dose, and the intensity of inflammation has been directly correlated with the intensity of fibrosis in a rabbit model (Shen *et al.,* 1988). IT instillation has the advantages of using less bleomycin than other methods, has a quicker kinetic development of lung injury and a clear time of initiation of injury. But the distribution of fibrosis is patchy with more peribronchial damage compared with administration via other routes.

Bleomycin induces lung injury by generating reactive oxygen radicals under aerobic conditions on binding to intracellular DNA and iron which in turn leads to DNA damage (Sugiura and Kikuchi 1978; Thrall and Scalise, 1995). This can then reduce molecular oxygen leading to generation of various types of reactive oxygen species that can cause marked DNA strand scission (Moseley, 1989).

2.4 Administration of bleomycin

Both male and female mice aged 6-10 weeks of age were used in all experiments. Animals were anaesthetised in a chamber using halothane gas (3%) in 2L/min O_2. On a heated mat (37°C), the necks were shaved and dampened with ethanol. A 1cm incision was made over the trachea and the tissue blunt dissected to reveal the trachea which was then cannulated. Animals were instilled with bleomycin sulphate (1-3mg/kg body weight; Kyowa Hakko, Slough, UK) dissolved in 50µl 0.9% saline or 0.9% saline alone as a control. The bleomycin was injected directly into the anterior trachea using a 1ml syringe with a 23 gauge needle. Three stitches were used to close the wound and the animal allowed to regain consciousness. At least 6 animals per group were analysed for biochemical and cytological assessment and at least 3 for histological assessment.

2.5 Bronchoalveolar lavage and collection of lung tissue

Each animal was terminally anaesthetised using an intraperitoneal injection of pentobarbitone sodium (200mg/ml; Sanofi Animal Health, Watford, UK) 3, 7,14, 28 or 84 days following bleomycin instillation. Following laparotomy and exanguination by severing the inferior vena cava, the trachea was cannulated with a 22 gauge venflon (Ohmeda BOC, Sweden). The lungs were lavaged with a total of 5ml PBS in ten 0.5ml aliquots. This maximised the total number of cells retrieved whilst avoiding over-inflation of the lung. Each aliquot was instilled slowly over 15 seconds, left *in situ* for 30 seconds and then removed over a further 15 seconds. The bronchoalveolar lavage fluid (BALF) was kept on ice throughout the procedure and over 90% of the instilled volume was consistently recovered. After lavage the trachea was cut and the lungs were subsequently removed, blotted dry on tissue and snap frozen in liquid nitrogen prior to analysis of lung hydroxyproline. The lavage samples were centrifuged (300 x g) at 4°C for 5 minutes to pellet the cells and the fluid removed and stored as 1ml aliquots at –80°C prior to analysis of PGE_2.

2.6 Cytological analysis of bronchoalveolar lavage

Following the removal of the BALF, the cell pellet was resuspended in 500µl Dulbecco's Modified Eagles Medium (DMEM, Gibco-BRL, Paisley, UK) containing 10% foetal bovine serum (FBS, Imperial Laboratories, Andover, UK) to a single-cell suspension. Total lavage cell numbers were determined using a haemocytometer on an inverted light microscope (Axioscope 20, Carl Zeiss, Germany). Cell preparations were generated from 100µl of single-cell suspension containing between 0.5-1.0 x 10^6 cells/ml and were spun in a cytometer (Cytospin 3, Shandon, UK) at 1000rpm for 3 minutes. The slides were left overnight to dry and then fixed in methanol and stained. Differential cell numbers were assessed by counting DiffQuik (DADE AG, Switzerland) stained cytospin preparations. At least 500 cells per sample were differentiated, using conventional morphological criteria for macrophages/monocytes, lymphocytes, and polymorphonuclear leukocytes (PMNs). Total cell numbers were expressed $x10^{-3}$ and differential cell counts as either total number of macrophage/monocyte, lymphocytes or PMNs $x10^{-3}$, or as a percentage of the total cell number.

2.7 Measurement of prostaglandin E_2 (PGE_2)

PGE_2 was measured in BALF, lung tissue and cell-conditioned medium using a specific enzyme immunoassay (Amersham Biosciences, Amersham, UK). The assay is based on competition between unlabelled PGE_2 and a fixed quantity of specific antibody. Once bound, the antibody complex is immobilized on pre-coated microtitre wells and the amount of labelled PGE_2 determined by adding a horseradish peroxidase enzyme substrate. The concentration of PGE_2 in unknown samples of both BALF and cell-conditioned media was determined by interpolation from a standard curve.

2.7.1 Assay protocol

The protocol used permitted the detection of PGE_2 in the range of 40 to 640pg/ml. Samples containing higher concentrations of PGE_2 were diluted accordingly in PBS (BALF) or serum-free DMEM (cell-conditioned media and lung tissue). The coated 96 well microtitre plate and reagents were thawed and equilibrated to room temperature before use. Assay buffer (0.1M phosphate buffer) was prepared by diluting 1M phosphate buffer in H_2O. This buffer was then used to reconstitute the lyophilized PGE_2 antibody and conjugate. The conjugate was stored on ice for the duration of the assay while the antibody remained at room temperature. Wash buffer (0.01 M phosphate buffer) was prepared by diluting 0.4M phosphate buffer in ddH_2O. A standard curve for PGE_2 was prepared by diluting a stock solution of PGE_2 in assay buffer with concentrations ranging from 1 to 32 pg/well.

All blanks and standards were run in duplicate. To determine non-specific binding (NSB) of the conjugate, 100µl assay buffer was added to the first two wells, followed by 50µl assay buffer into the zero standard wells. After vortexing, 50µl of each standard was pipetted into the appropriate well, starting with the most dilute. 50µl of the neat and diluted unknown samples were aliquoted into the remaining wells. Finally 50µl of PGE_2 antibody was added to all wells except the blanks and NSB wells and the plates covered and incubated on ice at 4°C for 3h. At the end of the incubation period, 50µl of conjugate was added to all wells except the blank. The plate remained on ice and was incubated for a further 1h. At the end of the incubation period, the contents of the plate were flicked out and the wells were filled with wash buffer using a wash bottle. This was repeated a further 3 times. After the plate was dried on blotting paper to remove residual buffer, 150µl of enzyme substrate, 3,3',5,5'-tetramethylbenzidine (TMB) was

added to each well and the colour reaction allowed to proceed at RT on a plate shaker for 30min. The reaction was terminated with the addition of 100µl 1M H_2SO_4 and the optical density (OD) immediately measured at 450nm, using a microtitre plate spectrophotometer (Titertek Multiscan).

2.7.2 Calculations

The average OD for each set of replicate wells was calculated. The NSB value was subtracted from the standards and unknown samples and the percentage bound substrate ($\%B/B_0$) determined using the following formula:

$$\%B/B_0 = \frac{\text{(standard or sample OD – NSB OD)}}{\text{(zero standard OD – NSB OD)}} \times 100$$

The standard curve was generated by plotting $\%B/B_0$ (y axis) as a function of the log PGE_2 concentration (x axis). A representative standard curve is shown in **Figure 2.3.** The concentration of PGE_2 in 50µl of unknown samples was read directly from the linear region of the sigmoid curve. Results were expressed as pg PGE_2 per ml BALF, or pg per 10^5 cells for *in vitro* experiments.

2.8 Measurement of 15-epi-Lipoxin A_4 (15-epi-LXA_4)

Similarly to PGE_2, 15-epi-LXA_4 was measured in BALF using a specific enzyme immunoassay (Neogen Corporation, supplied by Quidel, UK). The assay operates on a similar basis of competition between the enzyme conjugate and 15-epi-LXA_4 in the sample for a limited number of binding sites. The bound enzyme conjugate is detected by the addition of a coloured substrate and the extent of colour development is inversely proportional to the amount of 15-epi-LXA_4 in the sample, which is determined by interpolation from a standard curve. However, unlike PGE_2, 15-epi-LXA_4 can not be directly assayed in BALF and requires purification using solid-phase extraction prior to the assay.

2.8.1 Sample preparation

A 500µl aliquot of BALF was acidified to pH 3.5 with the addition of approximately 20µl 1N HCl. C_{18} Sep-Pak light cartridges (Waters Corporation) were used for extraction and preconditioned by washing with 2ml ethanol followed by 2ml ddH_2O.

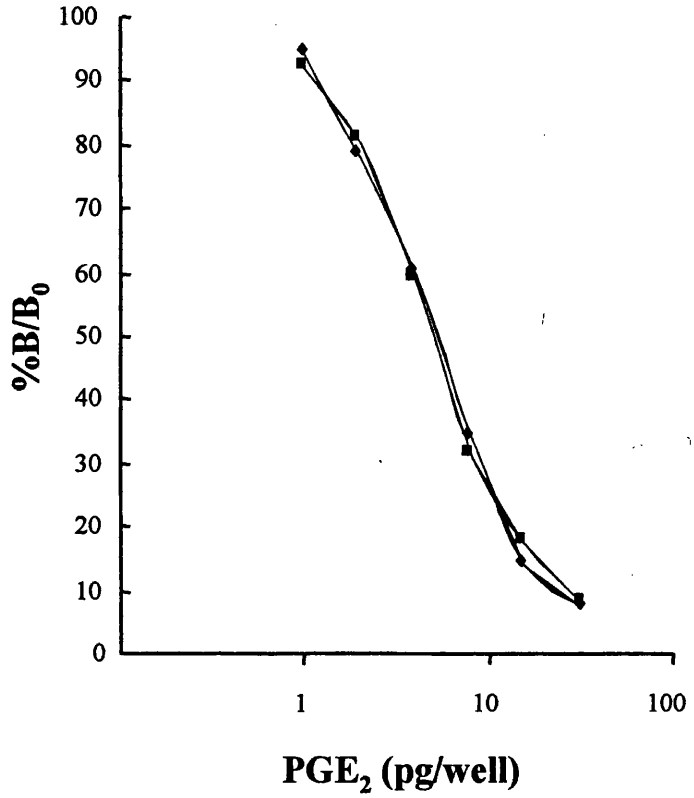


Figure 2.3 Representative prostaglandin E_2 standard curve
A stock solution of PGE_2 was serially diluted to known concentrations ranging from 1-32pg/well. A standard curve was generated by plotting $\%B/B_0$ as a function of the log PGE_2 concentration. Replicate data points are shown at each concentration. The concentration of PGE_2 in unknown samples was determined by interpolation from the linear region of the curve.

The acidified sample was applied to the column at a rate of 1ml per minute. Following this the column was washed with 1ml of ddH_2O followed by 1ml of petroleum ether. The 15-epi-LXA_4 was eluted with 2ml of methyl formate and the methyl formate was evaporated with a stream of nitrogen gas. The residue was dissolved in 250µl of diluted extraction buffer provided in the kit (diluted 1:5 in ddH_20) and assayed for 15-epi-LXA_4 content.

2.8.2 Assay procedure

The protocol permits the detection of 15-epi-LXA_4 in the range of 0.02-2ng/ml. Samples containing higher concentrations of 15-epi-LXA_4 were diluted accordingly in extraction buffer. Two vials of lyophilized horseradish peroxidase conjugate were provided and were each reconstituted using 75µl of ddH_2O. This was further diluted 1:50 using EIA buffer. If the contents of both vials of conjugate were required, the concentrated conjugates were pooled before dilution to the working concentration. Wash buffer provided was diluted 10-fold with ddH_2O. A 96 well microtitre plate was provided pre-coated with anti-15-epi-LXA_4 rabbit antibody. A standard curve for 15-epi-LXA_4 was prepared by diluting a stock solution of 15-epi-LXA_4 in EIA buffer with concentrations ranging from 0.02 to 2 ng/ml. Blanks and standards were run in duplicate. 50µl of EIA buffer was added to the zero standard wells and after vortexing, 50µl of each standard was pipetted into the appropriate wells, starting with the most dilute. 50µl of the diluted unknown samples were aliquoted into the remaining wells. Finally 50µl of dilute enzyme conjugate was added to each well except the blank, the plate was mixed on a microplate shaker and then covered and incubated at room temperature for one hour. At the end of the incubation period, the contents of the plate were flicked out and the wells were filled with wash buffer from a wash bottle. This was repeated a further two times as instructed. After the plate was dried on blotting paper to remove residual buffer, 150µl of enzyme substrate, stabilized 3,3', 5,5' Tetramethylbenzidine (TMB) plus hydrogen peroxide, was added to each well and the colour reaction allowed to proceed at RT on a plate shaker for 30mins. The plate was either read in a microtitre plate spectrophotometer (Titertek Multiscan) at 650nm or the reaction was stopped with 50µl of Neogen's Red Stop Solution and then read at 650nm.

2.8.3 Calculations

After the substrate background was subtracted from all absorbance values, the average OD for each set of replicate wells was calculated. The zero standard became the B_0 value and the percentage bound substrate ($\%B/B_0$) was determined using the following formula:

$$\%B/B_0 = (\text{standard or sample OD} / B_0) \times 100$$

The standard curve was generated by plotting $\%B/B_0$ (y axis) as a function of the log 15-epi-LXA_4 concentration (x axis). A representative standard curve is shown in **Figure 2.4**. The concentration of 15-epi-LXA_4 in unknown samples was read directly from the linear region of the sigmoid curve. Results were expressed as ng 15-epi-LXA_4 per ml BALF.

2.9 Measurement of Leukotriene C_4 (LTC_4)

LTC_4 was measured in BALF using a specific enzyme immunoassay (Cayman Chemical Company, supplied by Alexis Corporation, UK) and required solid-phase extraction prior to assay. C_{18} Sep-Pak light cartridges were used following a similar method to that described in **section 2.8**, substituting methanol for ethanol to increase solubility of LTC_4. Following evaporation of methyl formate, the residue was dissolved in 350µl of serum-free DMEM.

Again, the assay operates on the basis of competition between unlabelled LTC_4 and an LTC_4-acetylcholinesterase conjugate (LTC_4 tracer) for a limited amount of LTC_4 antiserum. The amount of LTC_4 tracer is determined by adding Ellman's Reagent, which contains acetylthiocholine and 5,5'-dithio-*bis*-(2-nitrobenzoic acid), the substrates for acetylcholinesterase. The concentration of LTC_4 in unknown samples was determined by interpolation from a standard curve. The protocol permitted the detection of LTC_4 in the range 7.8-1000pg/ml. EIA buffer was prepared by diluting the concentrate provided 1:10 using ddH_2O. This buffer was then used to reconstitute the lyophilized LTC_4-acetylcholinesterase tracer and LTC_4 antiserum. Wash buffer was prepared by diluting the concentrate provided (5ml) to a total volume of 2L with ddH2O with the addition of 1ml of Tween 20 provided.

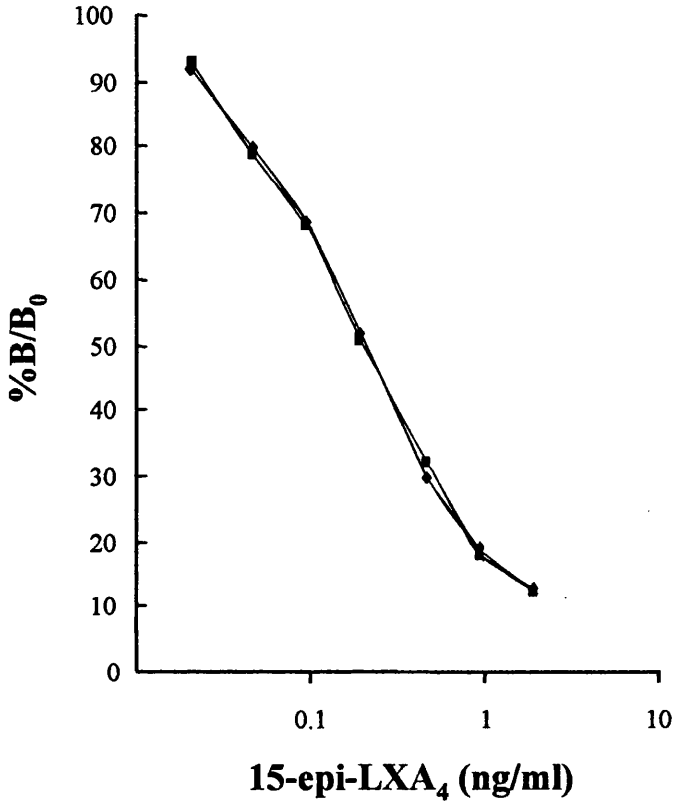


Figure 2.4 Representative 15-epi-lipoxin A_4 standard curve
A stock solution of 15-epi-LXA_4 was serially diluted to known concentrations ranging from 0.02-2ng/ml. A standard curve was generated by plotting $\%B/B_0$ as a function of the log 15-epi-LXA_4 concentration. Replicate data points are shown at each concentration. The concentration of 15-epi-LXA_4 in unknown samples was determined by interpolation from the linear region of the curve.

A standard curve for LTC_4 was prepared by diluting the LTC_4 standard using ddH2O to give a stock solution of 10ng/ml. A standard curve for LTC4 was prepared by further diluting the stock solution in EIA buffer to give concentrations ranging from 7.8-1000pg/ml. All blanks and standards were run in duplicate. To determine non-specific binding (NSB) of the tracer, 100µl assay buffer was added to the first two wells, followed by 50µl EIA buffer into Maximum Binding (B_0) wells. After vortexing, 50µl of each standard was pipetted into the appropriate well, starting with the most dilute. 50µl of the unknown samples were aliquoted into the remaining wells and 50µl of LTC_4 tracer was added to each well except the NSB and the blank wells. The plate was covered and incubated for 18h at room temperature. To develop the plate a vial of Ellman's reagent provided was reconstituted with 20ml of ddH_2O. This is light sensitive and was wrapped in aluminum foil. The contents of the plate were flicked out and the wells were filled with wash buffer using a wash bottle. This was repeated a further 4 times. After the plate was dried on blotting paper to remove residual buffer, 200µl of Ellman's Reagent was added to each well. The plate was covered in aluminum foil and the colour reaction allowed to proceed at RT on a plate shaker for 60-90 minutes. The plate was read at 405nm using a microtitre plate spectrophotometer.

2.9.1 Calculations

The average OD for each set of replicate wells was calculated. The NSB value was subtracted from the standards and unknown samples and the percentage bound substrate ($\%B/B_0$) determined using the following formula:

$$\%B/B_0 = \frac{(\text{standard or sample OD} - \text{NSB OD})}{(B_0\text{OD} - \text{NSB OD})} \times 100$$

The standard curve was generated by plotting $\%B/B_0$ (y axis) as a function of the log LTC_4 concentration (x axis). A representative standard curve is shown in **Figure 2.5**. The concentration of LTC_4 in unknown samples was read directly from the linear region of the sigmoid curve. Results were expressed as pg LTC_4 per ml BALF.

2.10 Histological assessment of lung fibrosis

Animals were terminally anaesthetized 28 days following bleomycin instillation as described (**section 2.5**). Following laparotomy and exanguination by severing the

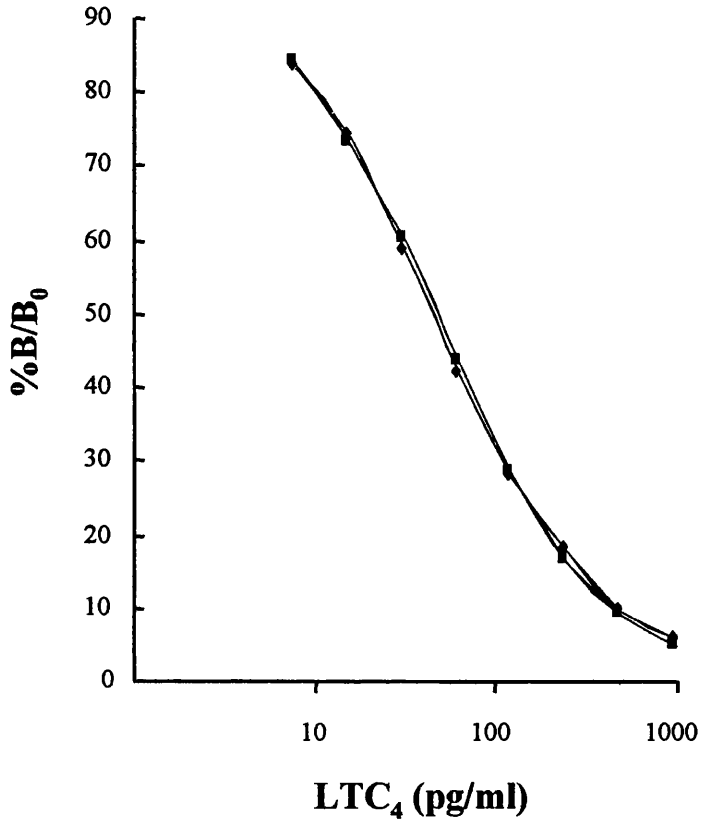


Figure 2.5 Representative leukotriene C_4 standard curve
A stock solution of LTC_4 was serially diluted to known concentrations ranging from 7.8-100pg/ml. A standard curve was generated by plotting $\%B/B_0$ as a function of the log LTC_4 concentration. Replicate data points are shown at each concentration. The concentration of LTC_4 in unknown samples was determined by interpolation from the linear region of the curve.

inferior vena cav , the pulmonary vasculature was perfused using approximately 2ml heparinised PBS by direct injection into the right atrium using a butterfly cannula until the lungs turned white. This was followed by 2ml of ice cold 4% paraformaldehyde to fix the lungs. The trachea was then cannulated using a 22 gauge venflon (Ohmeda BOC, Sweden) and inflated and fixed by intratracheal instillation of ice cold 4% paraformaldehyde fixative in PBS at a pressure of 20cm H_20 for 5 mins. The trachea was ligated and the heart and lungs removed *en bloc* and stored in 4% paraformaldehyde overnight at 4°C.

Tissue processing and slide staining was performed by Mr. Steve Bottoms.

The lungs were processed to paraffin wax and 3µm sections were cut and stained with a specific stain for matrix proteins, modified Martius Scarlet Blue. This is a trichrome stain in which there is serial application of Orange G, Red mix (Ponceau de Xylidine and acid fuchsin) and Chicago sky blue dyes in order to differentiate tissues. Staining was performed in an automated stainer (Sakura DRS-601, Bayer plc, Newbury, UK). Sections were firstly dewaxed and rehydrated through a series of solutions containing decreasing concentrations of alcohol. They were then treated with Lugol's Iodine to improve the differentiation of subsequent staining. Iodine was removed using sodium thiosulphate and sections were immersed in Celestine Blue to improve retention of haematoxylin. Sections were then treated with the first two trichromes, Orange G followed by Red Mix. Phosphotungstic acid was used to stabilise red staining before the third trichrome was applied, Chicago Blue. This was differentiated with acetic acid before the sections were dehydrated, cleared in xylene and coverslips were applied using an automated machine (Sakura Coveraid, Bayer plc, Newbury, UK). The full staining protocol is given in **Table 2.2**:

Step	Time	Treatment	Notes
1	3 min	Xylene	
2	3 min	Xylene	
3	2 min	100% alcohol	
4	2 min	90% alcohol	
5	2 min	70% alcohol	
6	5 min	Lugol's reagent	
7	3 min	Sodium thiosulphate	3% in distilled water
8	1 min	Tap water	
9	10 min	Celestine Blue	
10	1 min	Tap water	
11	30 sec	Distilled water	
12	5 min	Haematoxylin	
13	3 sec	Tap water	
14	20 sec	Acid alcohol	
15	2.5 min	Tap water	
16	30 sec	90% alcohol	
17	8 min	Orange G	0.2% in Picric alcohol
18	5 min	Distilled water	
19	7 min	Red mix	0.5% Ponceaux de Xylidine and 0.5% Acid Fuchsin in 1% Glacial acetic acid
20	20 sec	Distilled water	
21	30 sec	Phosphotungstic acid	
22	20 sec	Distilled water	
23	5 min	Chicago sky blue	
24	20 sec	1% acetic acid	
25	20 sec	70% alcohol	
26	20 sec	90% alcohol	
27	20 sec	100% alcohol	
28	4 min	Xylene	

Table 2.2 Modified Martius Scarlet Blue staining protocol

2.11 Measurement of hydroxyproline by reverse phase high pressure liquid chromatography (HPLC)

Hydroxyproline was measured as an index of total lung collagen, samples were processed according to previously published methods from our laboratory (Mutsaers *et al.,* 1998) Hydroxyproline was quantified by reverse-phase HPLC of 7-chloro-4-nitrobenzo-oxao1,3-diazole (NBD-Cl)-derivatized lung protein hydrosylates. The secondary amino acids hydroxyproline and proline react with NBD-Cl to generate a chromophore with maximum light absorbance at 495nm. Total lung collagen was calculated assuming that the lung contains 12.2% w/w hydroxyproline (Laurent *et al.,* 1981).

2.11.1 Tissue preparation

Lungs were removed 14, 28 or 84 days following instillation of either saline or bleomycin and snap frozen in liquid nitrogen as described in **section 2.5**. Lungs were stored at –80°C before being crushed to a fine powder under liquid nitrogen using a pestle and mortar. Aliquots of 10-20mg lung powder were accurately weighed and hydrolysed for 16 hours in 2mls 6M HCl at 110°C. Samples were then decolourised by the addition of approximately 20mg of charcoal (BDH, Merck, UK) and filtered (0.65μm pore, Millipore, Watford, UK). Samples were stored at room temperature prior to chromatography.

2.11.2 Amino acid derivatization

The samples were derivatised following methods previously published from our laboratory (Campa *et al.*, 1990). Briefly, the clear filtered hydrolysates were diluted 1:100 using ddH_2O and 200μl aliquots transferred to 1.5ml centrifuge tubes, three holes pierced in the lid, and evaporated under vacuum using a centrifugal sample concentrator (Savant, SpeedVac Plus SC110, Life Science International). The dried hydrolysate was re-dissolved in 100μl ddH_20, buffered with 100μl 0.4M potassium tetraborate, pH 9.5 and mixed with 100μl of 36mM NBD-Cl in methanol. Whilst minimising exposure to light, samples were vortexed and incubated in a water-bath at 37°C for 20 minutes. The reaction was stopped by adding 50μl of 1.5M HCl followed by 150μl of a concentrated solution of buffer A (167mM sodium acetate, pH 6.4, in 26% acetonitrile v/v). After vortexing and filtering through a HPLC 0.22μm pore size filter (Sartorius), 100μl aliquots were injected into the HPLC column and eluted with an acetonitrile gradient.

2.11.3 Chromatographic conditions

Derivatised samples were separated using a single pump HPLC system (HPLC system 523, Biotek Instruments, Bucks, UK) with a reverse-phase cartridge column (LiChroCART LiChrospher 250mm length x 4mm diameter, 5μm particle size, 100 RP-18, BDH/Merck) following previously published methods from our laboratory (Campa *et al.*, 1990). Running buffers A (8% v/v acetonitrile and 50mM sodium acetate, pH 6.4) and B (75% v/v acetonitrile) were freshly prepared and degassed (Gastorr 153, Biotek Instruments) prior to use. The HPLC system was equilibrated in buffer A for 40 minutes and then three derivatized standard solutions containing 50pmol hydroxyproline were analysed. Derivatized standards and samples were eluted at 1ml/min though an

acetonitrile gradient, generated by changing the relative proportions of buffer A and B over time as shown below:

Time	% Buffer B
0	0
5	5
6	80
12	80
12.5	0
25	0

Table 2.3 Elution gradient for separation of hydroxyproline by reverse-phase HPLC

The running time per sample was 25 minutes and hydroxyproline produced a peak at approximately 5-6 minutes. **Figure 2.6** shows a representative chromatograph of a standard and a sample of lung tissue.

2.11.4 Quantification of lung collagen content

Amounts of hydroxyproline (hyp) were quantified by comparing peak areas with those generated from a standard solution prepared under identical conditions to produce an 'HPLC number'. This number was used to calculate total lung collagen (mg/lung) using the following calculation:

$$\text{HPLC number} \times \frac{\text{Volume of HCl (2000µl)}}{\text{Volume of dried hydrolysate (200µl)}} \times \text{Dilution factor (100) of hydrolysate} \times \text{Dilution factor of sample (5)}^{a} \text{ injected onto column}$$

$$= \text{pmol of hyp} \times \left(\frac{131^{b}}{1000000000}\right) = \text{mg hyp} \times \frac{\text{lung wt}}{\text{aliquot wt}} \times 8.1967^{c} = \text{mg collagen per lung}$$

Note:

a. *Dilution factor is 5 since the redissolved sample is diluted 1:5 by the addition of 0.4M potassium tetroborate, NBD-Cl, HCl and concentrated solution of buffer A.*

b. *131 represents the molecular weight of hydroxyproline*

c. *8.1967 converts mg hyp per lung to mg collagen/lung by assuming the lung contains 12.2% w/w hyp, where 8.1967 = 100% / 12.2% (Laurent et al., 1981)*

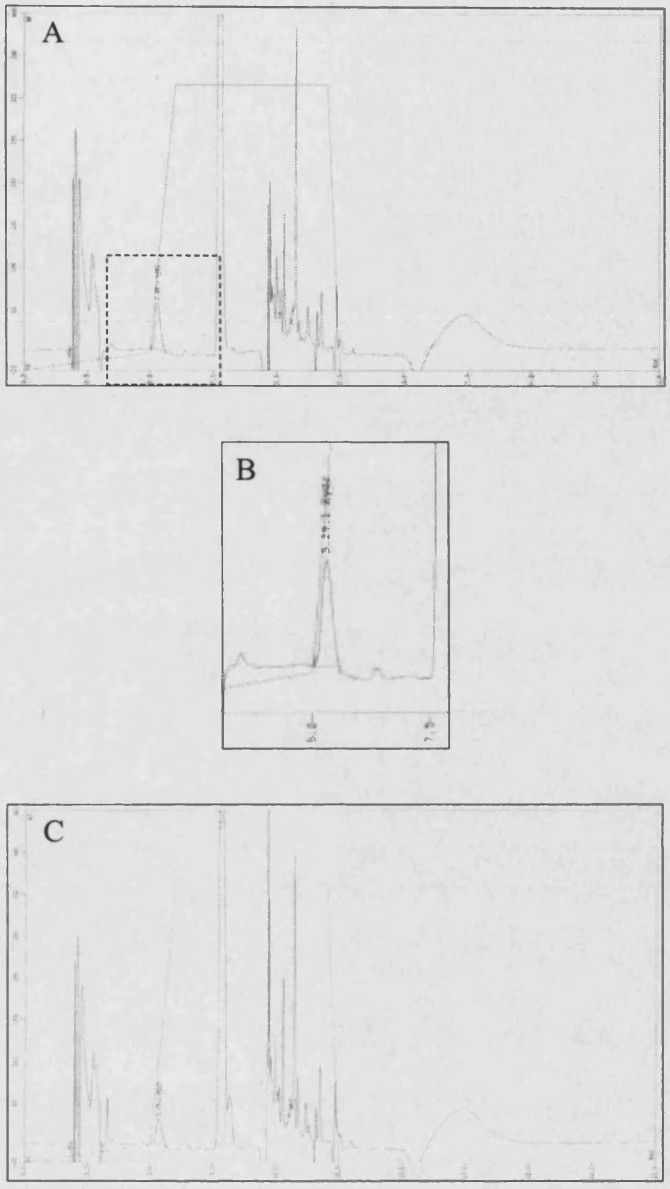


Figure 2.6 Representative HPLC chromatographs
Figures 2.6A and 2.6B show a reprentative chromatograph of a hydroxyproline standard of known concentration eluted at 5.29mins. The area under the hydroxyproline curve shown in Figure 2.6B is used to calculate the amount of hydroxyprlone present in an unknown sample of lung hydroslyate shown in Figure 2.6C

2.12 Western Analysis of COX-1, COX-2 and $cPLA_2$ protein expression

COX-1, COX-2 and $cPLA_2$ protein expression was examined using western analysis of lung tissue taken 7 and 14 days following instillation of bleomycin.

2.12.1 COX-2 protein expression

Approximately 10-20μg lung tissue removed either 7 or14 days following instillation of bleomycin or saline was homogenised in 500μl of buffer (50mMTrisHCl, 0.1% Tween20) containing protease inhibitors (Complete, Roche, Germany) using a glass Ultraturis homogeniser. The homogenate was centrifuged (12000 x g) at 4°C for 30mins to pellet tissue fragments and the supernatant analysed. Protein was measured in a 5μl aliquot of each sample using a bicinchoninic acid (BCA) colourmetric assay (Pierce, UK) to ensure equal quantities were applied to each lane. Working reagent was prepared by combining 50 parts reagent A ($NaCO_3$, $NaHCO_3$ BCA detection reagent and $C_6H_4O_6Na_2$ in 0.2N NaOH) with 1 part reagent B (4% w/v $CuSO_4$ solution). 200μl of the working reagent was added to 5μl of either sample or known protein standards, and 5μl of homogenising diluent and incubated for 30 minutes at 37°C. The absorbance was read at 550nm using a microtitre plate spectrophotometer (Titertek Multiscan).

7.5% SDS-PAGE gels were constructed and electrophoresed using the XCell II Sure Lock™ Mini Cell system (Novex, San Diego, USA). Each gel contained one lane of 5μl molecular weight marker (Rainbow™, Amersham Pharmacia Biotech, UK) and one lane of 100ng of purified COX-2 (ovine) electrophoresis standard (Cayman Chemicals/Alexis Corporation, Nottingham, UK). Sample lysate containing 25-30μg protein was added to 8x Laemlli sample buffer and heated at 95°C for 5mins. The lysates were pulse centrifuged for 30 seconds at 10000 x g before equal amounts of protein (25-30μg) were loaded onto the remaining lanes and the gel run for 2-3 hours at 150V in SDS-PAGE tank buffer (25mM Tris, 192mM glycine and 0.1% w/v SDS). Two gels were run, one gel was used for western analysis and the companion gel stained with 0.1% v/w Coomassie brilliant blue R-250, 10% v/v methanol and 4% acetic acid for 2 hours and then destained using a solution of 10% v/v methanol and 7.5% v/v acetic acid. The remaining gel was submerged in transfer buffer (SDS-PAGE tank buffer, 10% methanol) and proteins electro-transferred onto a nitrocellulose membrane (Hybond-ECL, Amersham Pharmacia Biotech, UK) for 2hrs at a constant current of $0.8mA/cm^2$ using semi-dry blotting apparatus (BioRad, UK). Following transfer, non-specific

binding was blocked by incubating the membrane for 1-2 hours in 5% skimmed milk (Marvel, UK) in TBST (50mM Tris, 150mM NaCl, 0.02% Tween-20®, pH 7.4) at RT. The membrane was incubated overnight at 4°C with a COX-2 polyclonal rabbit anti-mouse primary antibody (Cayman Chemicals/Alexis Corporation, Nottingham, UK) at a 1:1000 dilution in 2% skimmed milk TBST. Following 3 x 5 mins washing in TBST, the membrane was incubated in a horseradish peroxidase conjugated goat anti-rabbit secondary antibody at a dilution of 1:2000 in 2% skimmed milk TBST for 1 hour at room temperature. The membrane was washed again for 3 x 15 mins in TBST, and then immunoreactive bands visualised using enhanced chemiluminescence (ECL) western blotting detection agents (Amersham Pharmacia Biotech, UK). Band intensities were calculated by scanning the films (Sharp JX-300 colour scanner) and quantifying band densities using Image Master™ software (Amersham Pharmacia Biotech, UK).

2.12.2 COX-1 and cPLA$_2$ protein expression

Dr Caroline Wheeler-Jones *at the Royal Veterinary College, London*, performed the majority of western blots for cPLA$_2$ and COX-1 in lungs removed 14 days following instillation of either saline or bleomycin.

Protein was extracted from lung tissue using a technique of repeated freeze-thaws. Approximately 20µg of powdered lung tissue was added to 200µl RIPA buffer (1% Triton X-100 (w/v), 0.1% SDS (w/v), 158mM NaCl, 1mM EDTA, 10mM Tris-HCl, 1mM $NaVO_3$, 1mM AEBSF, 50µg/ml leupeptin, pH 7.2) and immersed in liquid nitrogen for several seconds. This was followed by immersion in a 37°C water-bath for several seconds. Samples were vortexed every second freeze-thaw cycle. This cycle was repeated 5 times per sample. Samples were then centrifuged (5min, 10000 x g, 4°C) and the supernatants transferred to clean microcentrifuge tubes. Protein was measured as described above and lysate containing 100-150µg protein was added to 2x Laemlli sample buffer and heated at 95°C for 5mins. The lysate was pulse centrifuged for 30 seconds at 10000 x g before being loaded into lanes of a 10% SDS-PAGE gel. Gels were constructed and electrophoresed using the PROTEAN II xi electrophoresis equipment (BioRad, Hemel Hemstead, UK). Each gel also contained one lane of a molecular weight marker (New England BioLab, USA). Purified COX-1 and cPLA$_2$ protein standards were not used, however initial experiments contained thrombin-stimulated human umbilical vein endothelial cell (HUVEC) lysates prepared by Dr

Wheeler-Jones. These samples had previously been validated to produce immunoreactive bands consistent with both $cPLA_2$ and COX-1.

Gels were electrophoresed at a constant current of 25mA per gel as samples moved through the stacking gel, followed by 15mA per gel overnight for 20 hours in SDS-PAGE tank buffer (25mM Tris, 192mM glycine and 0.1% w/v SDS). Following electrophoresis, gels were submerged in transfer buffer and proteins electro-transferred onto PVDF membrane for 3hrs at a constant current of $0.8mA/cm^2$ using semi-dry blotting apparatus (BioRad, UK). To examine protein loading, the gel was subsequently stained with a solution of 0.1% v/w Coomassie brilliant blue R-250, 10% v/v methanol and 4% acetic acid for 2 hours and then destained using a solution of 10% v/v methanol and 7.5% v/v acetic acid.

The membrane was carefully cut into two at approximately 80kDa, as visualised by the molecular weight markers (New England BioLab, USA). This allowed the top section of the gel to be probed for $cPLA_2$ at 110 kDa and the lower part COX-1 at 70kDa. Non-specific antibody binding was blocked by incubating the membrane for 3 hrs in 0.2% w/v I-Block® dissolved in TBS (20mM Tris, 137mM NaCl, pH 7.6) for $cPLA_2$, and 3% BSA in TBST for COX-1. Membranes were incubated overnight at room temperature with the appropriate primary antibody (COX-1 – goat anti-rabbit polyclonal, and $cPLA_2$ – rabbit anti-mouse monoclonal antibodies, both used 1:1000 (both Santa Cruz, Calne, Wiltshire UK)) diluted in TBST. Blots were then washed with TBST (6 x 10min) and incubated for 1 hr with horseradish-peroxidase (HRP) conjugated anti-goat IgG (COX-1) or anti-rabbit IgG ($cPLA_2$) both 1:10000 dilution in TBST. Following further washing (8 x 10min) in TBST, immunoreactive bands were visualised by ECL according to manufacturers instructions, and band densities quantified as previously described.

Section 3 *In vitro* studies

2.13 Wild type and COX-$2^{-/-}$ fibroblast PGE_2 production

2.13.1 Isolation of murine lung fibroblasts

Lungs were removed from untreated WT and COX-$2^{-/-}$ mice aged 6-8 weeks and transferred to the laboratory in a 50ml centrifuge tube containing serum–free DMEM

containing 4mM L-glutamine, 100U/ml penicillin, 100µg/ml streptomycin and 2.5µg/ml amphoteracin B. The lungs were cut into small pieces with a sterile scalpel in a petri dish and transferred into a 50ml centrifuge tube containing 20ml DMEM and 1mg/ml Type 2 bacterial collagenase (Worthington Biochemical Corporation, New Jersey, USA). The tissue was incubated at 37°C for 2 hours in a shaking water-bath. After this time the suspension was filtered through a 100µm nylon filter (Falcon, Becton Dickinson, USA) to remove debris and diluted at a ratio of 1:1 with FBS to reduce the collagenase activity. The suspension was then centrifuged at 300 x g for 5 minutes to pellet the cells and resuspended in 20% FBS DMEM. The cell suspension was then re-centrifuged to remove any remaining bacterial collagenase and then suspended in 5ml DMEM containing 20% FBS, 4mM L-glutamine, 100U/ml penicillin, 100µg/ml streptomycin and 2.5µg/ml amphoteracin. The cell suspension was transferred to a $25cm^3$ tissue culture flask (TRP, Switzerland) and incubated in a humidified atmosphere of air containing 10%CO_2 at 37°C. Cell culture media was replaced every 3-4 days. Cells were routinely cultured in $75cm^3$ tissue culture flasks. Amphotericin B was included for the initial three passes only and cultures of COX-2 deficient fibroblasts were also supplemented with 1µg/ml indomethacin (Sigma) for the first three passages

2.13.2 Cell passage

Cells were passaged when confluent. Media was removed and the cell monolayer washed with 10ml PBS to remove traces of serum. 5mls of trypsin/EDTA (trypsin 0.05% w/v; EDTA 0.02% w/v) was added to the monolayer and the cells were incubated for 2 minutes at 37°C. When visualised using an inverted light microscope (Axioscope 20, Carl Zeiss, Germany) the cells became rounded and detached from the culture flask. An equal volume of DMEM containing 10% FBS was added to the flask to neutralise the trypsin. This 10mls was removed and centrifuged at 300 x g for 5 minutes to pellet the cells. The media was removed and the cell pellet resuspended in 10mls DMEM containing 20% FBS, glutamine and antibiotics with a 1:4 split ratio.

2.13.3 Measurement of PGE_2 in cell-conditioned media

Wild type and COX-$2^{-/-}$ fibroblasts were used for measurement of PGE_2 at passage numbers 2-4. Once confluent, cells were trypsinized as previously described (**section 2.13.2**). Cells were resuspended in 20% FBS DMEM and seeded at 10^5 cells/well in 12 well plates and grown to confluence. Once confluent, cells were incubated for a further 24 hours. The media was removed and replaced with 1ml preincubation media

containing 0.4% (v/v) FBS, 4mM glutamine, 50μg/ml ascorbic acid and 0.2mM proline for 24 hours. After this time the preincubation media was replaced with 1ml fresh media containing either 0.2% FBS (control media), or control media with TGF-β_1 (1ng/ml), NS398 (5μg/ml), or both TGF-β_1 and NS398 and incubated for a further 24 hours. The media was then removed and frozen at -80°C before being analyzed for PGE_2 as previously described (**section 2.7**). The cell layer was then trypsinized using 500μl trypsin and 500μl of DMEM containing 10% FBS to give a 1ml single-cell suspension and the cell number counted using an Improved Neubauer Haemacytometer (BDH/Merck) under phase-contrast using an inverted light microscope. The number of cells /ml was then calculated using the formula:

$$\text{Cells/ml} = \text{number of cells counted} \times 10^{-4}$$

Fibroblast PGE_2 production was expressed as pg/10^5 cells.

2.14 Wild type and COX-$2^{-/-}$ macrophage/monocyte PGE_2 production

Due to the difficulty in isolating sufficient pulmonary macrophages, an established model routinely used in the laboratory was used to isolate peritoneal macrophages/monocytes (Kruisbeek & Vogel, 1995; Bellingan *et al.*, 2002). WT and COX-$2^{-/-}$ mice aged 6-8 weeks were injected intraperitoneally (I.P) with 2ml (4%w/v) thioglycolate. Bellingan and co-workers have previously shown macrophage/monocyte numbers to be maximal 5 days following I.P injection of thioglycolate, with minimal numbers of PMNs at this time (Bellingan *et al.*, 2002). Therefore, at day 5 the animals were killed by cervical dislocation and the peritoneum lavaged using 5ml sterile PBS. Lavage fluid was kept on ice at all times. The inflammatory cells were isolated by centrifuging the lavage fluid (300 x g) for 5 minutes at 4°C. The cells were resuspended in serum free DMEM and counted using a haemacytometer as described above. Cells were seeded at 1000 cells/well in 96 well plates and left overnight to adhere in a humidified atmosphere of air containing 10%CO_2 at 37°C. The cell layer was washed 3 times with DMEM to remove red blood cells and non-adherent inflammatory cells. 150μl fresh media was then added to each well and the plate incubated for a further 24 hours in a humidified atmosphere of air containing 10%CO_2 at 37°C. After 24 hours the media was removed and assayed for PGE_2 as previously described in **section 2.7**.

2.15 Statistical analysis

All *in vivo* data are presented as mean ± standard error of the mean (SEM) for at least 6 animals unless stated. *In vitro* data is presented as mean ± SEM for 6 replicate cultures and is representative of 3 separate experiments. Statistical analysis was performed using an unpaired Students t-test for single, or ANOVA for multiple group comparison. A p value of less than 0.05 was considered significant.

CHAPTER THREE:

RESULTS

Section 1. Preliminary studies to determine optimum bleomycin dose and timepoints for studies of inhibition of COX-2 function

3.1 Effect of sex on body and lung weight following intratracheal instillation of saline and bleomycin

Due to limited numbers of mice available, performing all animal experiments using only one sex of mouse was not feasible. As expected, the mean body weight of male animals (WT, COX-$2^{+/-}$ and COX-$2^{-/-}$ in equal proportions) was significantly greater than female mice 14 days following administration of either saline or bleomycin (**Figure 3.1A**, $p<0.001$). To overcome the variation in body weight between male and female animals, the sexes were equally distributed throughout all experiments described. Furthermore, sex did not affect lung weight 14 days following administration of either saline or bleomycin (**Figure 3.1B**), and therefore differences in body weight between male and female animals are unlikely to have a discernible affect on BALF PGE_2 levels or total lung collagen production.

3.2 Wild type mice

Due to the breeding strategy of mating COX-$2^{+/-}$ males with COX-$2^{+/-}$ females, COX-$2^{+/+}$ (wild type, WT) littermate animals were produced in addition to the strain-matched WT mice originally provided by the suppliers, and used by this laboratory in a previous publication (Keerthisingham *et al.*, 2001). Both strains of wild type mice are on a C57BL6/SV129 background and represent equally valid controls since littermate mice have also been used in previously published work with COX-2 deficient mice (Zeldin *et al.*, 2001). Both control groups are represented in this thesis due to the availability of the mice; strain matched animals were used in the majority of studies undertaken at earlier periods, whereas the work progressed to using wild type littermate animals once these became freely available. The first section will discuss phenotypic differences between the wild type strains in respect to body weight and their response to bleomycin injury. This will be characterised by parameters fundamental to this thesis, i.e., levels of PGE_2 in bronchoalveolar lavage (BALF) and total lung collagen content.

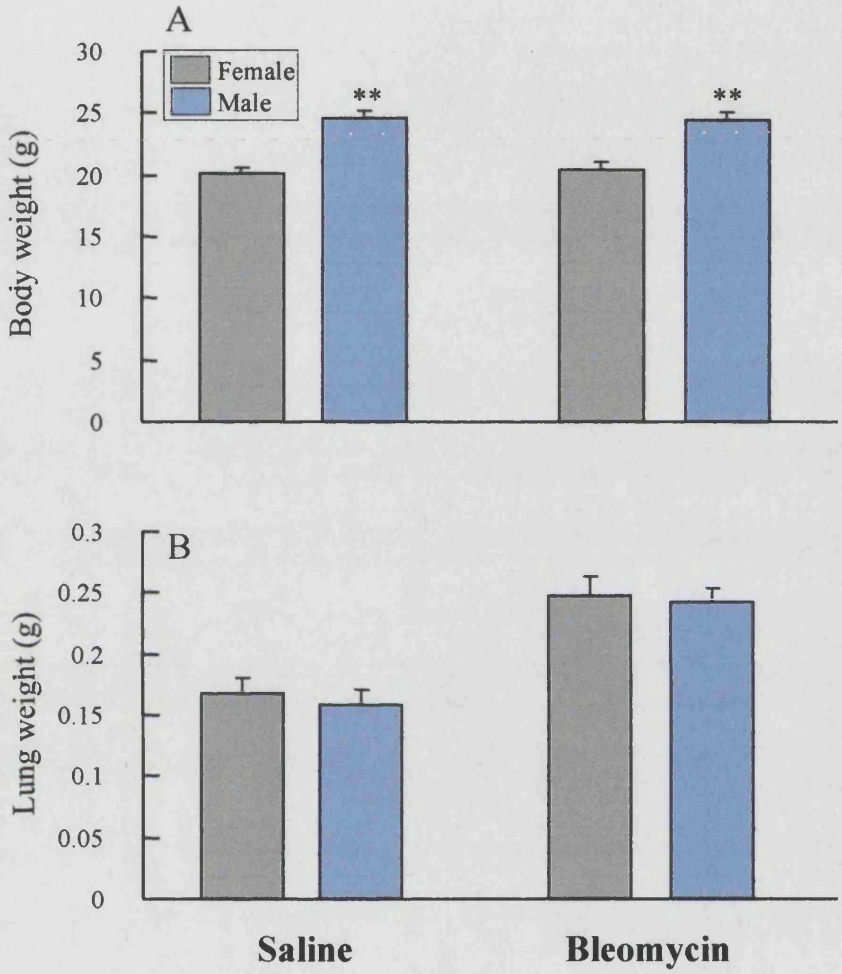


Figure 3.1 Effect of sex on body and lung weight 14 days following instillation of either saline or bleomycin

Figure 3.1A shows female and male body weight (equal distribution of all strains and COX-2 genotype animals) in grams 14 days following instillation of either 0.9% saline or bleomycin (1mg/kg). Figure 3.1B shows male and female lung weight in grams at this time. 10-16 B196 mice (WT, COX-$2^{+/-}$ and COX-$2^{-/-}$ mice in equal proportions) at 6-8 weeks of age were used in each group. ** indicates $p<0.001$ compared with female mice.

3.2.1 *Body weight and weight loss following intratracheal instillation of bleomycin in wild type mice*

Figure 3.2A shows the average body weight of both littermate (B196) and strain-matched (B197) control mice at 6-8 weeks of age prior to use. No difference was observed between the initial starting weight of either strain of wild type animals. **Figure 3.2B** represents changes in body weight over 28 days following IT administration of either saline or bleomycin (1mg/kg). At all timepoints examined, neither strain showed a significant difference in body weight following administration of bleomycin compared with saline alone. However, there were differences in the pattern of weight changes between the strains.

Littermate controls (B196) initially lost weight 3 days following instillation of saline alone however by day 7 body weight was greater than day 0 and continued to increase over the timecourse. By 28 days B196 body weight following saline alone was significantly greater than 3 days, $p<0.05$. Following bleomycin, B196 mice lost on average over 2g in weight by day 3 (approximately 10% of initial start weight), however again by day 7 this had been regained and body weight was significantly greater than day 3, $p<0.001$. B196 mice continued to gain weight over the timecourse and by day 28 had gained 5.45g compared with day 3, $p<0.005$.

Following administration of either saline or bleomycin, strain-matched wild type mice (B197) had lost weight at day 7 compared with day 0, however, since data is unavailable for B197 mice at day 3, it is unclear as to whether a greater loss in weight was observed at an earlier time. After day 7, body weight increased over the remaining timecourse, but at a lower rate than B196 mice and at day 28 body weight was not significantly different compared with the initial start weight. At days 7, 14 and 28 the weight change observed in B196 mice following instillation of saline was significantly greater than B197s, $p<0.01$ and at days 14 and 28 the weight change in B196s following bleomycin was significantly greater than B197s, $p<0.001$.

3.2.2 *PGE_2 production in wild type mice*

Day 14 was chosen as a representative timepoint to examine both BALF PGE_2 production and total lung collagen following injury, as significant increases in total lung collagen following bleomycin would not be expected prior to this time. Lungs were lavaged using PBS and PGE_2 measured in lavage fluid from both littermates and strain-

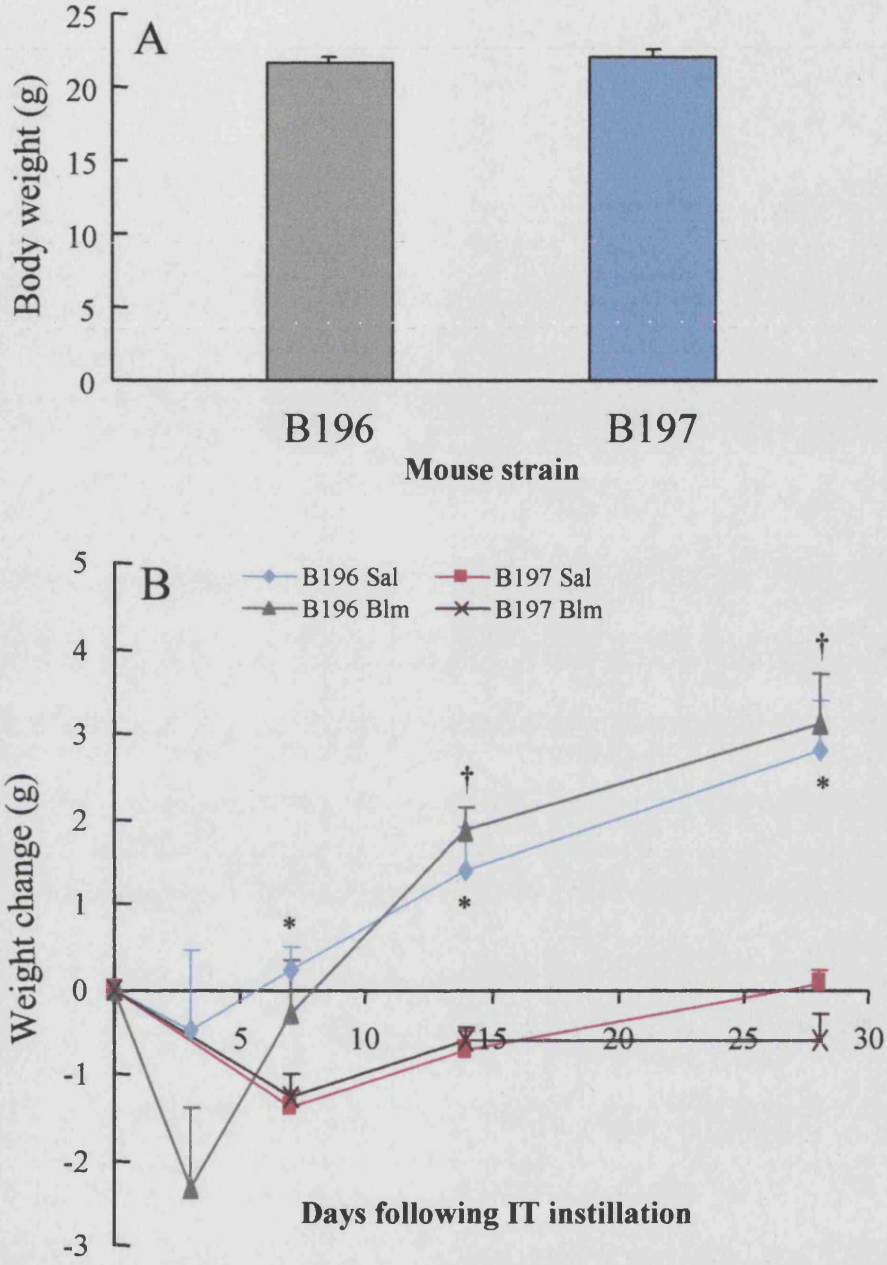


Figure 3.2. Comparison of body weight and weight change between strains of wild type animals following intratracheal instillation of saline or bleomycin

Figure 3.2A shows average starting body weight in grams in B196 and B197 at 6-8 weeks old. N= 20 mice per group. Figure 3.2B shows weight change following IT instillation of either 0.9% saline or bleomycin (1mg/kg). 6-8 animals were used in each group. * indicates $p<0.01$ compared with B197 Sal, † indicates $p<0.001$ compared with B197 Blm.

matched wild type mice. **Figure 3.3** shows that whilst both strains of WT mice showed a significant increase in BALF PGE_2 following injury, littermate animals (B196) produced significantly higher levels of BALF PGE_2, both basally, and following instillation of bleomycin compared with strain-matched controls (B197, $p<0.05$ in both cases). However the fold increase in BALF PGE_2 production following instillation of bleomycin was similar between strains (B196 6.6-fold, B197 7-fold).

3.2.3 Collagen production in wild type mice

At day 14 following instillation of either saline or bleomycin lungs were removed and collagen content was assessed by the measurement of hydroxyproline using HPLC as described (**section 2.11**). **Figure 3.4** shows that following instillation of saline alone collagen levels are similar in both strains of WT mice (B196: 1.58mg ±0.11 vs B197: 1.64mg ±0.09). Following the administration of bleomycin, total lung collagen increased significantly in both strains, as is typical of the bleomycin model (B196: 2.10mg ±0.14 vs B197: 2.34mg ±0.12, $p<0.05$ in both cases) although levels were not significantly different between the two strains.

In summary, both strains of wild type mice represent valid controls although some differences do exist in their phenotypes and their production of PGE_2 in the lung. For these reasons the results of the two strains were kept separate throughout all experiments, and not combined. All following data using COX-2 deficient mice will be compared to only one strain, which will be clearly identified.

3.3 Bleomycin dose response

Increasing doses of bleomycin were used to investigate the effect on PGE_2 and collagen production in the model.

3.3.1 Effect of bleomycin dose and PGE_2 production

Previous studies in mice have shown that levels of PGE_2 in lung homogenates (Giri *et al.*, 1985) and in serum (Chandler *et al.*, 1983) are increased following intratracheal instillation of bleomycin. Additionally, **Figure 3.3** shows a significant increase in BALF PGE_2 following IT administration of bleomycin (1mg/kg) in both wild type control groups. **Figure 3.5** shows PGE_2 levels following increasing doses (0-3mg/kg) of bleomycin in strain-matched wild type mice (B197), 14 days following injury. There

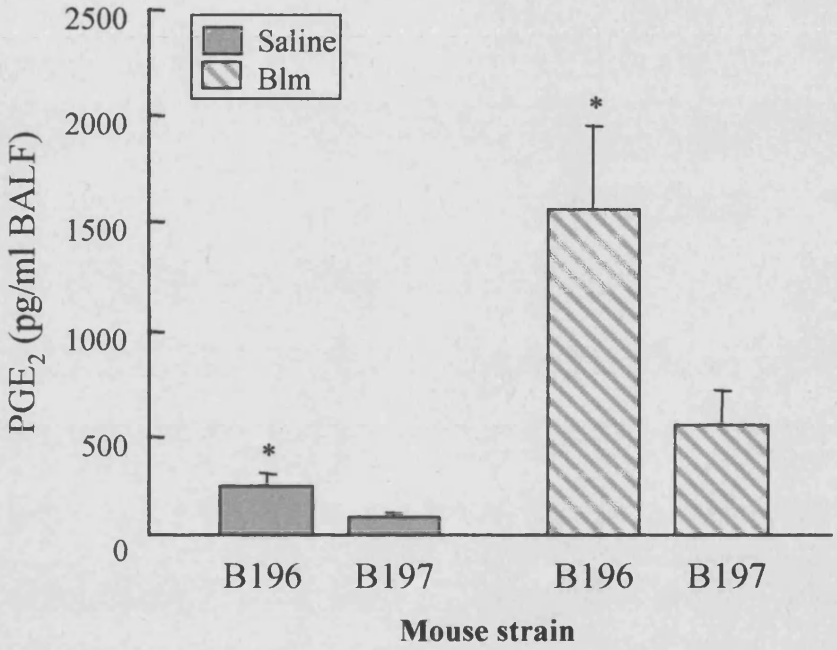


Figure 3.3. Comparison of BALF PGE_2 levels in strain-matched (B197) and littermate (B196) controls

Lungs were lavaged with PBS 14 days following IT administration of either saline or bleomycin (1mg/kg). BALF PGE_2 was measured by EIA. 7-10 animals were used per group.* indicates $p< 0.05$ compared with B197 equivalent treatment.

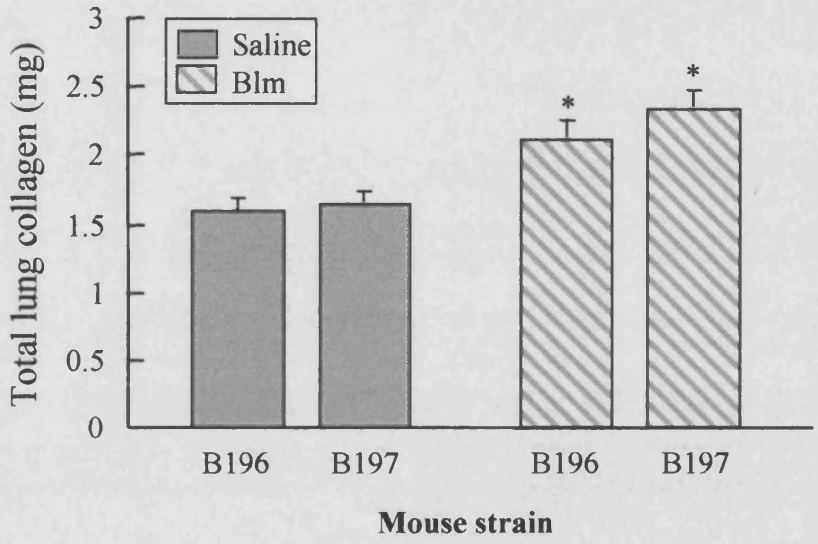


Figure 3.4. Comparison of total lung collagen accumulation in strain-matched (B197) and littermate (B196) controls
Lungs were removed 14 days following IT administration of either saline or bleomycin (1mg/kg) and total lung collagen measured by HPLC. 7-10 animals were used per group. * indicates $p< 0.05$ compared with strain-matched saline-treated mice.

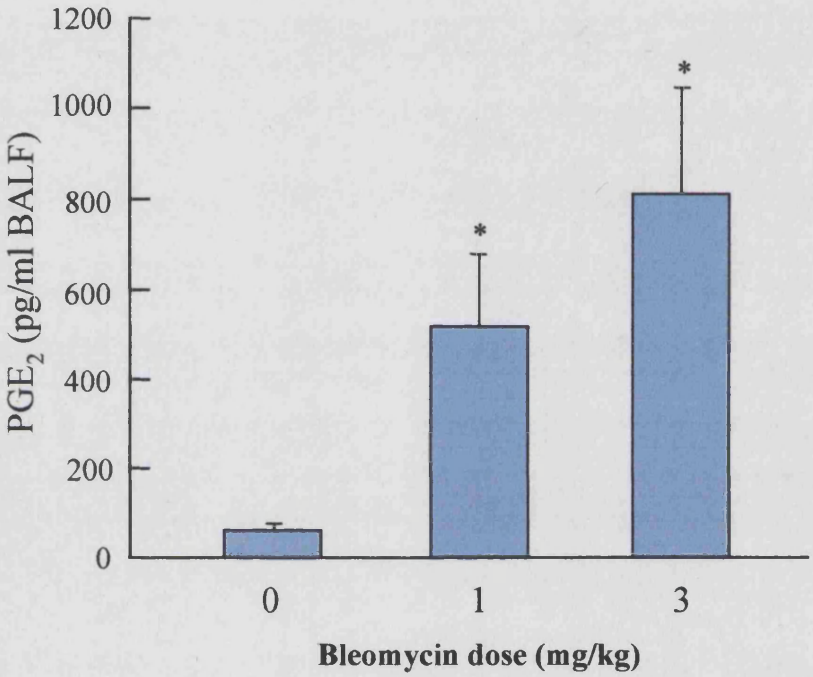


Figure 3.5. BALF PGE_2 levels following instillation of increasing doses of bleomycin

Animals were lavaged with PBS 14 days following instillation of bleomycin (0-3mg/kg). BALF PGE_2 was measured using a commercially available EIA. * $p<0.05$ compared with instillation of saline alone. 7-9 animals were used per group.

was an eight-fold and thirteen-fold increase in BALF PGE_2 levels following instillation of 1mg/kg and 3mg/kg bleomycin respectively, however the difference in PGE_2 produced between 1 and 3 mg/kg bleomycin was not statistically significant.

3.3.2 Effect of bleomycin dose on total lung collagen production

Figure 3.6 shows the significant increase in total lung collagen content in B197 wild type mice 14 days following IT administration of 1mg/kg bleomycin, as previously described (**Figure 3.4**, $p<0.05$). However, when the dose of bleomycin was increased to 3 mg/kg no further increase lung collagen content was observed .

Together, **Figures 3.5** and **3.6** show no significant differences in either BALF PGE_2 levels or lung collagen content using bleomycin at either 1 or 3 mg/kg. From the hypothesis, it is proposed that inhibition of COX-2 activity, either by pharmacological inhibition, or by using COX-2 deficient mice, will result in an enhanced fibrotic response to bleomycin compared with control animals. Although PGE_2 and collagen production do not alter significantly with the high dose of bleomycin, the low dose of 1mg/kg was chosen for all future experiments to maximise any potential increase in the fibrotic response seen in mice in the absence of COX-2 activity.

3.4 Effect of bleomycin on COX expression

Figures 3.3 and **3.5** show that bleomycin induces production of PGE_2 in the lung. To investigate which COX isoform(s) is (are) responsible for PGE_2 synthesis following bleomycin-induced lung injury, COX isoform protein expression was examined using western blotting. Strain-matched wild type mice were used at 14 days following instillation of bleomycin. **Figure 3.7** shows that protein expression of COX-1 increased approximately 1.5-fold following instillation of bleomycin compared with saline alone, although this increase is non-significant. In contrast, COX-2 protein expression is significantly upregulated following the instillation of bleomycin compared with saline alone, $p<0.05$.

Thus, it has been shown that in wild type mice bleomycin injury upregulates COX-2, but not COX-1 protein, which suggests COX-2 to be responsible for the significant increase in PGE_2 synthesis observed. As is typical of the model, the dose of bleomycin chosen for all future studies causes a significant increase in total lung collagen at 14

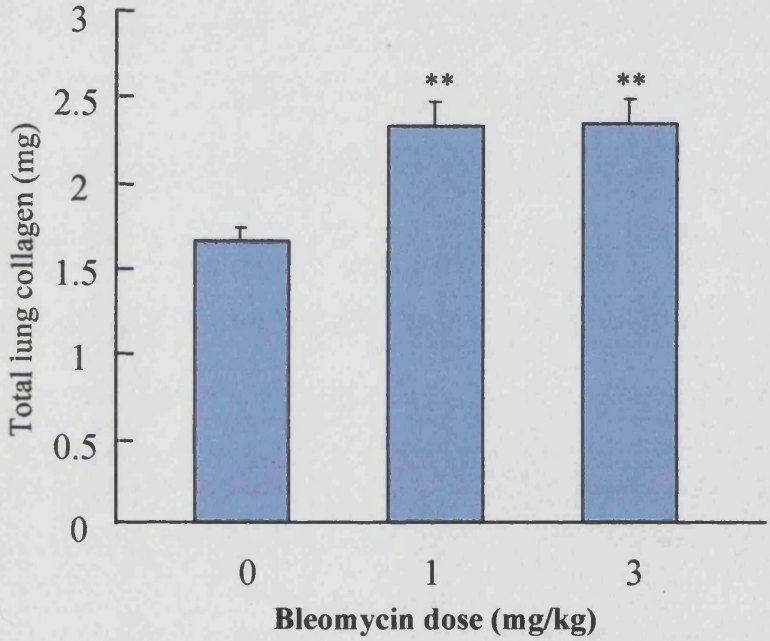


Figure 3.6 Effect of increasing doses of bleomycin on total lung collagen

Lungs were removed 14 days following IT administration of bleomycin (0-3mg/kg) and total lung collagen measured by HPLC. 7-9 animals were used in each group. ** indicates $p<0.01$ compared with saline alone.

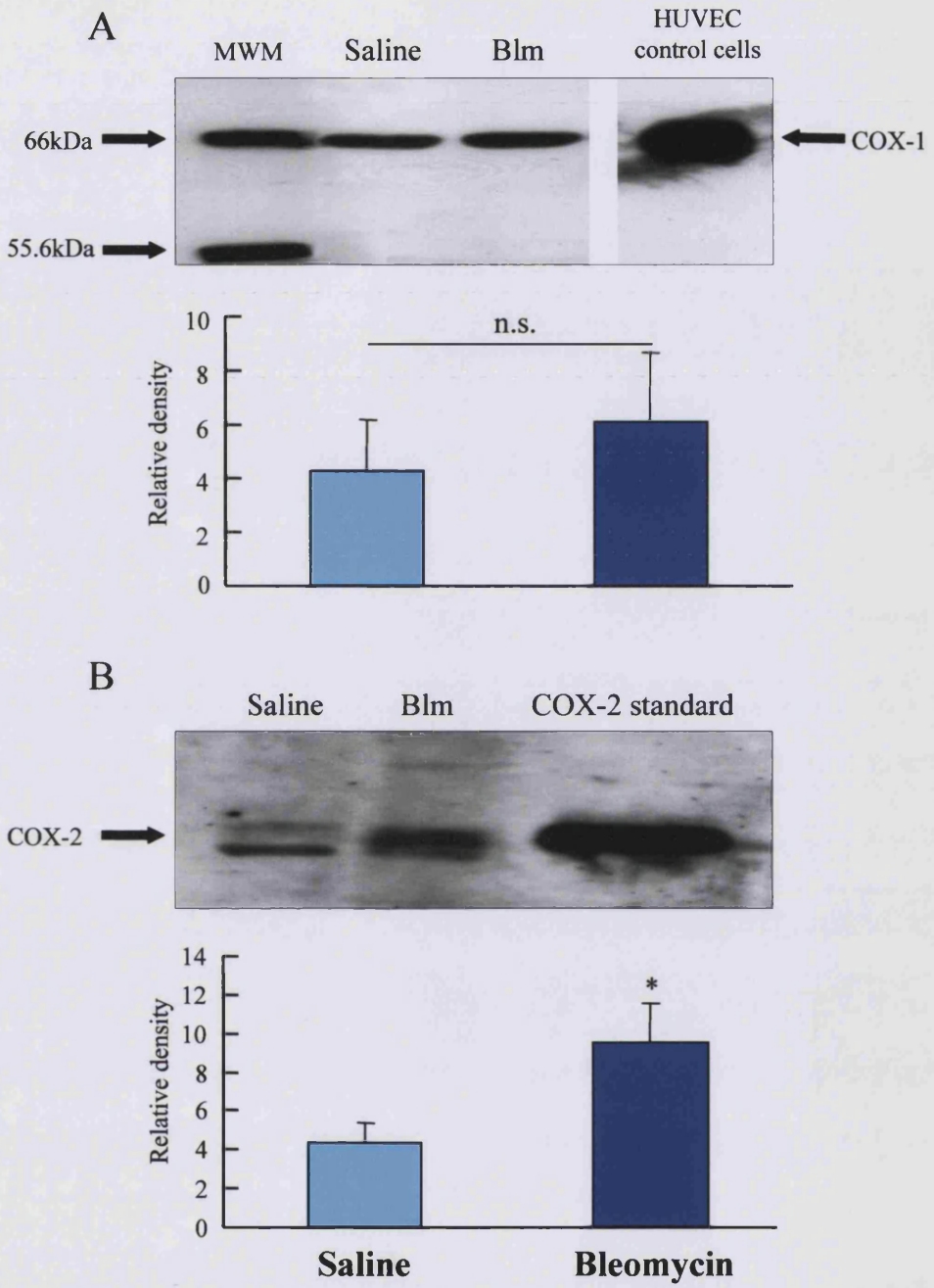


Figure 3.7. COX expression following IT administration of saline and bleomycin

Lungs were removed 14 days following injury. Protein was extracted and electrophoresed on SDS-PAGE gels. Levels of COX-1 and COX-2 were detected using western blotting with goat polyclonal COX-1 (**Figure 3.7A**) and rabbit polyclonal COX-2 antibodies (**Figure 3.7B**). Band densities were quantified as described and represent the mean ± SEM for 5 (COX-2) or 6 (COX-1) animals. * indicates $p<0.05$ compared with saline. MWM denotes molecular weight marker.

days following injury. These data suggest this model to be ideal for investigating the effects of inhibiting COX-2 activity *in vivo*.

Section 2. Pharmacological inhibition of COX-2

To further investigate if the induction of PGE_2 synthesis in the bleomycin model of pulmonary fibrosis is COX-2 specific, and the role of COX-2 in the development of the fibrotic response, mice were treated with the selective COX-2 inhibitor NS398 twice daily via oral gavage. B197 animals were used in all experiments throughout Section 2.

One hour prior to IT administration of either bleomycin (1mg/kg) or saline, animals were dosed with either NS398 (3mg/kg, see **sections 1.18, 2.3** and **4.3.1**) or vehicle (0.1%DMSO, 1%Tween80 in PBS). Subsequent doses of NS398 or vehicle were given every 12 hours thereafter.

3.5 Effect of instillation of NS398 on body weight

Figure 3.8 shows that administration of NS398 did not affect body weight at any time over the 28 day timecourse. Similarly to **Figure 3.2**, there is also no significant difference in weight loss in mice who received bleomycin compared with those who received saline.

3.6 Effect of NS398 on PGE_2 synthesis

Following treatment with either 3mg/kg NS398 or vehicle for up to 28 days, BALF PGE_2 levels were measured as previously described. **Figure 3.9** shows that following instillation of saline alone, BALF PGE_2 levels are low but measurable in all groups, and do not change with either time or treatment with NS398. This suggests that basal levels of BALF PGE_2 are COX-1-mediated.

Following IT administration of bleomycin and vehicle alone, BALF PGE_2 production significantly increased for at least 28 days following injury compared with saline control, $p<0.05$ in all cases (**Figure 3.9**). The highest levels were observed at the earliest timepoint examined, day 7 (744.3pg/ml BALF), and decreased in a time

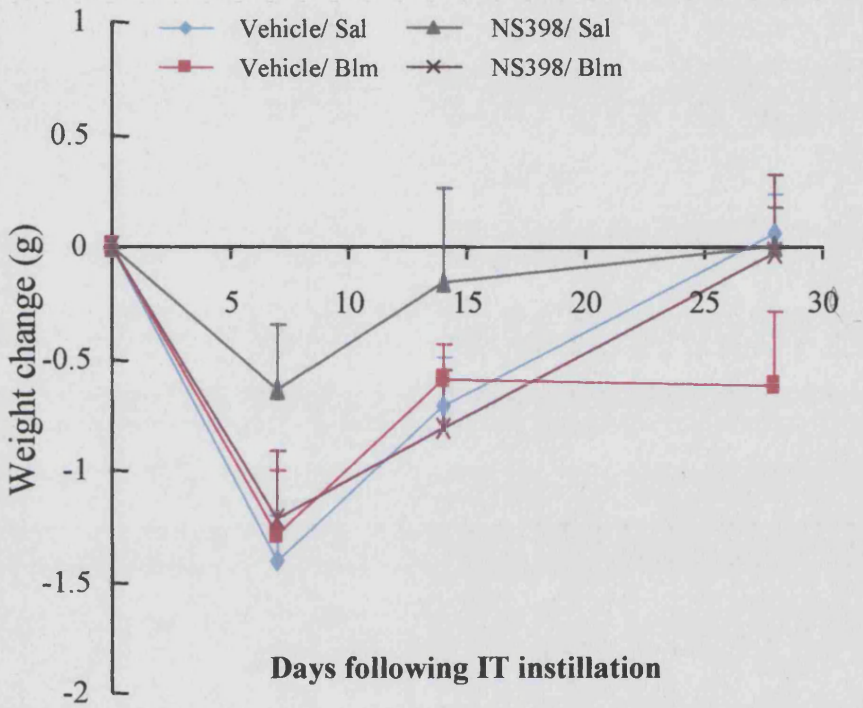


Figure 3.8. Changes in body weight following intratracheal instillation of saline or bleomycin and treatment with NS398
Following intratracheal instillation of either 0.9% saline or bleomycin (1mg/kg) animals were instilled twice daily via oral gavage with either NS398 (3mg/kg body weight) or vehicle (0.1%DMSO in PBS, 1% Tween80). 6-8 animals were used in each group.

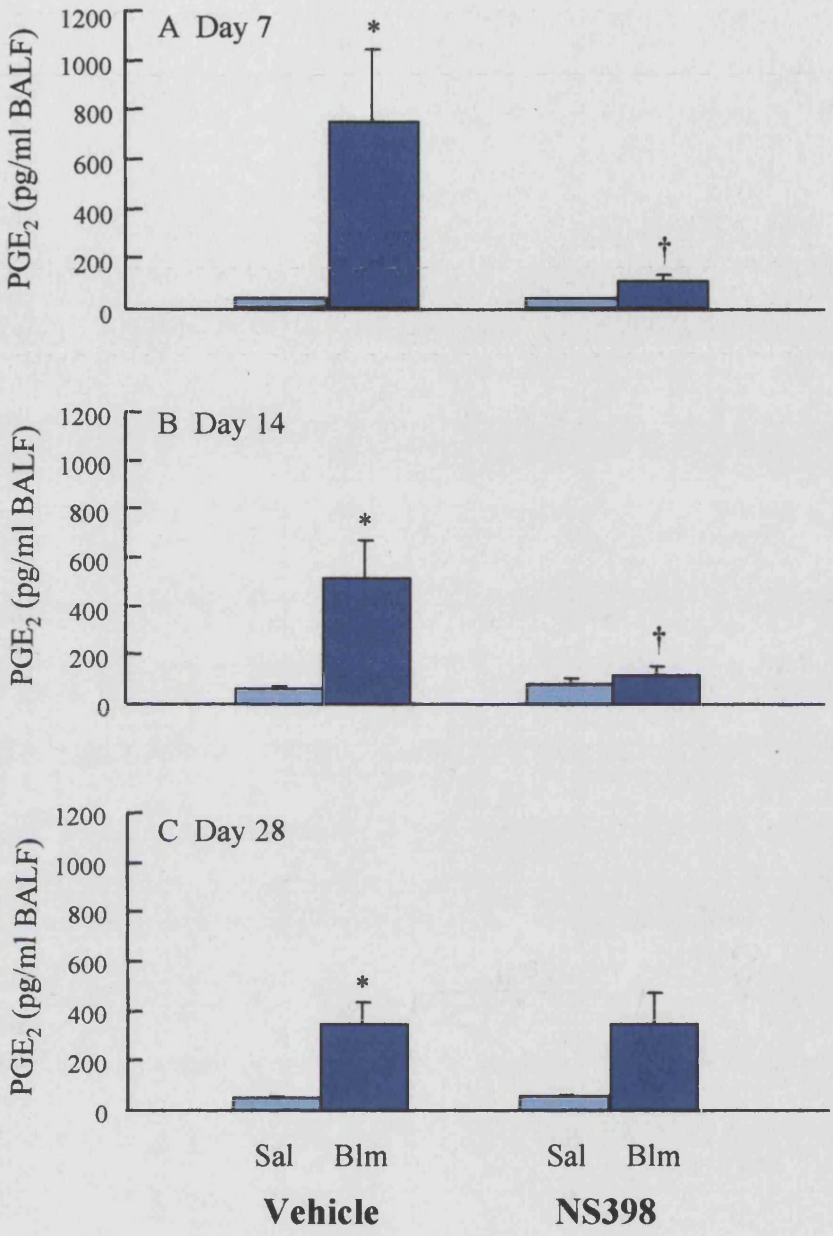


Figure 3.9. Effect of selective inhibition of COX-2 on PGE_2 production in the lung

Following treatment with either the selective COX-2 inhibitor, NS398, or vehicle, animals were lavaged using PBS at 7, 14 and 28 days following IT administration of either saline or bleomycin (1mg/kg). BALF PGE_2 was measured by EIA. 6-8 animals were used per group. * indicates $p<0.05$ compared with saline control, † $p<0.05$ compared with vehicle treated bleomycin.

dependent manner at days 14 (517.3pg/ml) and 28 (350pg/ml). However, at day 28 BALF PGE_2 was still significantly increased following instillation of bleomycin compared with saline alone, $p<0.05$.

At days 7 and 14 following instillation of bleomycin, treatment with the COX-2 selective inhibitor NS398 almost completely inhibited bleomycin-induced BALF PGE_2 production. At 7 days PGE_2 production was reduced by 86% and at 14 days by 77% compared with treatment with vehicle alone, and at both timepoints, values were not significantly different from animals treated with saline/NS398.

However, at 28 days bleomycin-induced PGE_2 production was no longer inhibited following administration of NS398. Levels of BALF PGE_2 in bleomycin/NS398-treated mice were similar to those in bleomycin/vehicle-treated animals (313.6 vs 350pg/ml BALF).

3.7 Effect of pharmacological inhibition of COX-2 on the development of bleomycin induced pulmonary fibrosis

NS398 has been shown to successfully inhibit PGE_2 production for at least 14 days following bleomycin instillation. PGE_2 is known to inhibit lung fibroblast collagen production (McAnulty *et al.,* 1995), and promote breakdown of newly synthesised collagen (Baum *et al.,* 1980; Brilla *et al.,* 1995), therefore it was hypothesised that pharmacological inhibition of COX-2 activity, and the subsequent inhibition of PGE_2 biosynthesis would lead to an enhanced fibrotic response to bleomycin.

Figures 3.10 shows that following treatment with vehicle alone, total lung collagen production significantly increases following instillation of bleomycin, $p< 0.05$. However total lung collagen content following bleomycin injury is not further enhanced following pharmacological inhibition of COX-2 using NS398. Indeed, there is no significant increase in the mean total lung collagen content in animals instilled with bleomycin compared with instillation of saline alone. At 28 days following instillation of bleomycin total lung collagen content is significantly increased compared with saline alone in both NS398 and vehicle-treated animals, $p<0.05$ in both cases, **Figure 3.11**. By 28 days lung collagen content had not increased further in vehicle-treated mice

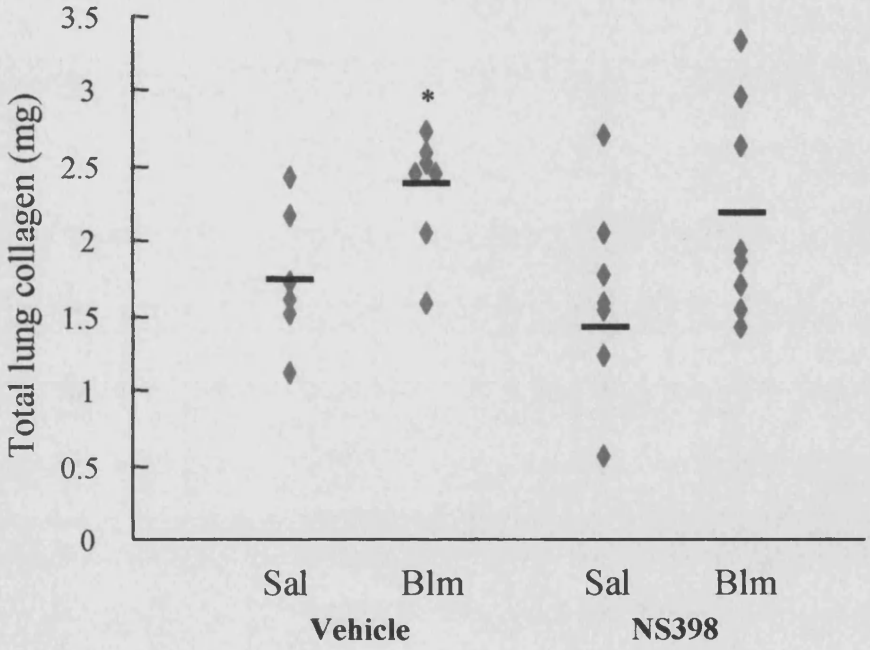


Figure 3.10. Effect of pharmacological inhibition of COX-2 on the fibrotic response to bleomycin 14 days following injury
Lungs were removed and total lung collagen measured using HPLC. Each point represents total lung collagen for an individual mouse. Mean lung weight per group is indicated by a horizontal bar. 6-8 animals were used per group. * indicates $p<0.05$ compared with respective saline control.

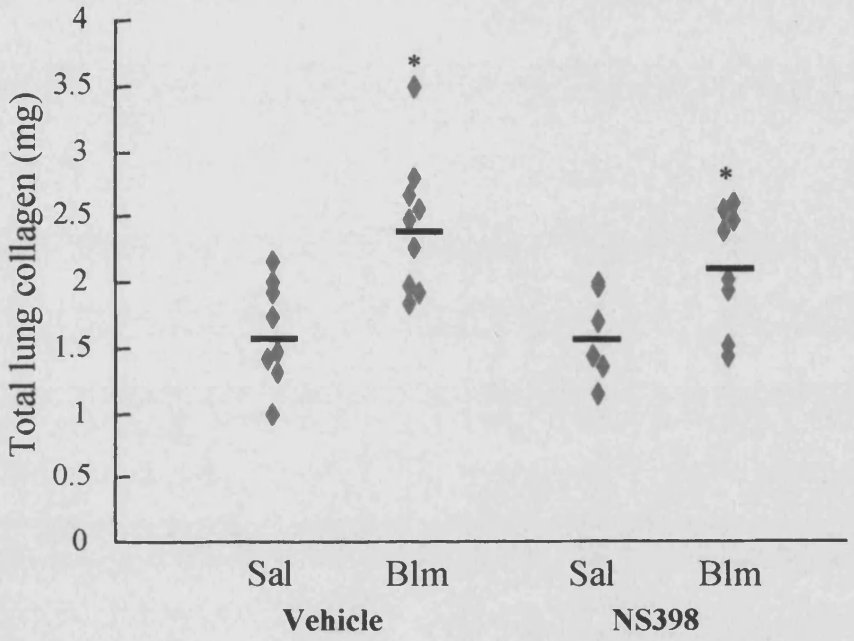


Figure 3.11. Effect of pharmacological inhibition of COX-2 on the fibrotic response to bleomycin 28 days following injury
Lungs were removed and total lung collagen measured using HPLC. Each point represents total lung collagen for an individual mouse. Mean lung weight per group is indicated by a horizontal bar. 7-9 animals were used per group. * indicates $p<0.05$ compared with respective saline control.

following administration of bleomycin compared with the equivalent group at day 14 (see **Figure 3.10**) following injury. Similarly, there was no further increase in lung collagen content between NS398-treated mice 28 days following bleomycin compared with those at day 14, and selective inhibition of COX-2 did not lead to an enhanced fibrotic response to bleomycin injury at this time compared with vehicle treated animals.

3.8 Effect of NS398 on inflammatory response to bleomycin-induced lung injury

At 7, 14 or 28 days following instillation of bleomycin, lungs were lavaged with PBS and inflammatory cells isolated. Total cell number was determined using a haemocytometer and cytospin preparations were made to obtain differential cell counts.

At days 7, 14 and 28 there was no difference in total cell numbers following bleomycin injury, compared with administration of saline alone. Furthermore, this was unaffected by treatment with NS398 at any timepoint examined (**Table 3.1**).

Following instillation of saline, BALF contained greater than 97% macrophages/monocytes at all timepoints examined, and was unaffected by treatment with NS398 (**Table 3.1**). In comparison, day 7 following instillation of bleomycin BALF contained a decreased proportion of macrophage/monocytes due to increased numbers of polymorphonuclear leukocytes (PMNs) and lymphocytes compared with saline alone, $p<0.001$ in all cases. However treatment with NS398 had no significant effect on the cellular profiles at day 7. Numbers of PMNs and lymphocytes present in BALF following instillation of bleomycin declined with time in both NS398- and vehicle-treated animals and by day 14 there was no longer a significant increase following bleomycin instillation compared with saline alone. In summary, treatment with NS398 did not affect the inflammatory response to bleomycin.

Day	I.T. instillation	Treatment	Total cell count (x10^3)	Macrophage, %	Neutrophil, %	Lymphocyte, %
7	Saline	Vehicle	383.5 ± 51.4	98.2 ± 0.5	0.1 ± 0.1	0.9 ±0.4
	Bleomycin	Vehicle	353.2 ± 40.8	76.7 ± 5.9 *	10.8 ± 2.9 *	12.8 ±3.0 *
	Saline	NS398	318.2 ± 43.1	97.9 ± 0.8	0.7 ± 0.3	1.5 ±0.5
	Bleomycin	NS398	406.2 ± 30.7	79.1 ± 1.7 *	7.1 ± 0.9 *	13.7 ±1.0 *
14	Saline	Vehicle	342.7 ± 60.1	98.4 ± 11.6	0.3 ± 0.9	1.2 ± 1.2
	Bleomycin	Vehicle	356.4 ± 34.5	95.8 ± 2.0	3.0 ± 1.8	1.0 ± 0.3
	Saline	NS398	281.4 ± 25.7	99.4 ± 0.2	0.2 ± 0.1	0.4 ± 0.2
	Bleomycin	NS398	386.5 ± 40.5	94.6 ± 2.1	3.4 ± 1.7	1.6 ± 0.5
28	Saline	Vehicle	292.3 ±34.1	99.1 ± 0.5	0.3 ± 0.1	0.6 ± 0.4
	Bleomycin	Vehicle	306.1 ±36.4	98.0 ± 0.6	1.6 ± 0.5	0.4 ± 0.1
	Saline	NS398	237.3 ±26.7	99.9 ± 0.1	0.1 ± 0.0	0.1 ± 0.0
	Bleomycin	NS398	331.4 ±37.2	98.2 ± 0.7	1.4 ± 0.4	0.4 ± 0.3

Table 3.1 Effect of NS398 on inflammatory response to bleomycin-induced lung injury

Following treatment with either the selective COX-2 inhibitor NS398 or vehicle alone, animals were lavaged using PBS at 7, 14 and 28 days following IT administration of either 0.9% saline or bleomycin (1mg/kg). Total cell number was determined using a haemocytometer and expressed as total cell count ($x10^{-3}$). Cytospin preparations were made to obtain differential cell counts which are expressed as a percentage of total cells. 6-8 animals were used per group. * indicates $p<0.001$ compared with relative treatment saline at day 7.

3.9 Histological assessment of bleomycin-induced lung injury following treatment with NS398

Lung injury was assessed at 14 and 28 days following instillation of bleomycin or saline in mice treated with either NS398 or vehicle control. Histological sections were stained using modified MSB where matrix proteins such as collagen are stained blue, and inflammatory cells stained red. Representative areas are shown in **Figures 3.12** and **3.13. Figure 3.12A** and **B** show animals treated with either vehicle or NS398 14 days following instillation of saline alone. In both groups, normal lung architecture is retained, with thin alveolar walls and matrix staining limited to the interstitium, airways and blood vessels. A small number of inflammatory cells are present in the interstitium, but administration of NS398 does not appear to greatly affect normal lung architecture. This does not alter after 28 days administration of NS398, where lung histology remains normal, and comparable to vehicle treated animals following saline instillation (**3.13A** and **B**).

In contrast, all mice instilled with bleomycin showed characteristic lesions (**Figures 3.12 and 3.13 C, D, E** and **F**) with patchy loss of alveolar architecture and thickening of the interstitium. This was accompanied by increased numbers of fibroblast /myofibroblast-like cells within the lesion (**Figures 3.12** and **3.13 E** and **F**). The increased matrix staining correlated with increased production of collagen in the lungs as assessed by the measurement of hydroxyproline (**Figure 3.10** and **3.11**). BALF inflammatory cell numbers were not increased following bleomycin injury at either 14 or 28 days (**Table 3.1**), however at a higher power, both **Figures 3.12E, F** and **3.13E, F** show significant numbers of inflammatory cells localised to lesions, irrespective of treatment with NS398.

3.10 Pharmacological inhibition of COX-2: Summary

Through western analysis and the use of the selective COX-2 inhibitor NS398, it has been shown that following bleomycin-induced lung injury, wild type mice synthesise PGE_2 predominantly through upregulation of COX-2. PGE_2 is capable of inhibiting several processes thought to be important in the fibrotic response, namely fibroproliferation, collagen production and myofibroblast differentiation. Therefore it was hypothesised that reduced levels of PGE_2, via inhibition of its biosynthesis, would

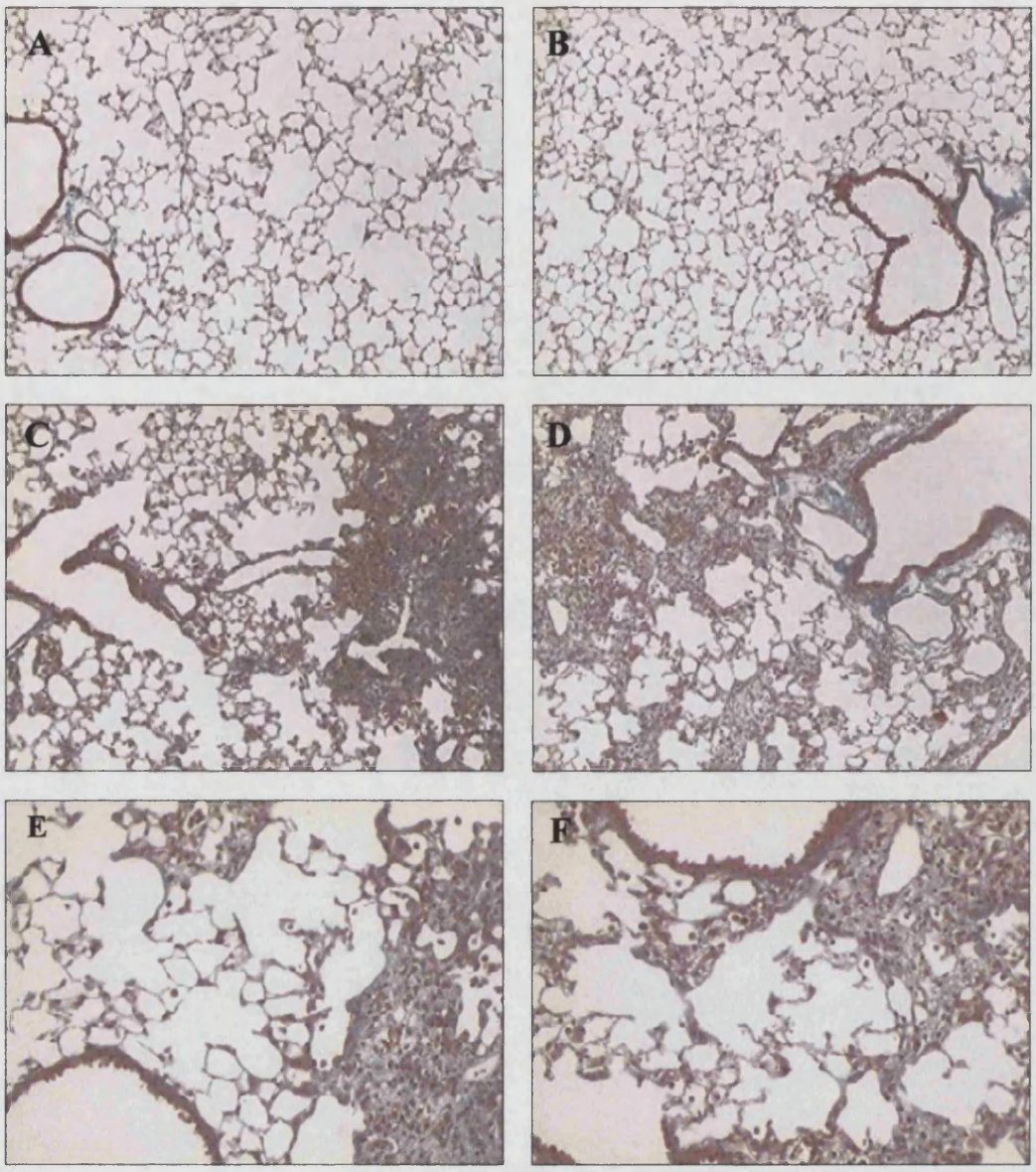


Figure 3.12. Effect of pharmacological inhibition of COX-2 using NS398 (3mg/kg) 14 days following IT instillation of either 0.9% saline or bleomycin (1mg/kg)

Micrographs of lung sections stained using modified MSB. A) vehicle treated and B) NS398-treated mice following instillation of saline alone. C) & E) vehicle-treated, D) & F) NS398-treated mice following instillation of bleomycin. Original magnification x100 (A-D) and x200 (E & F).

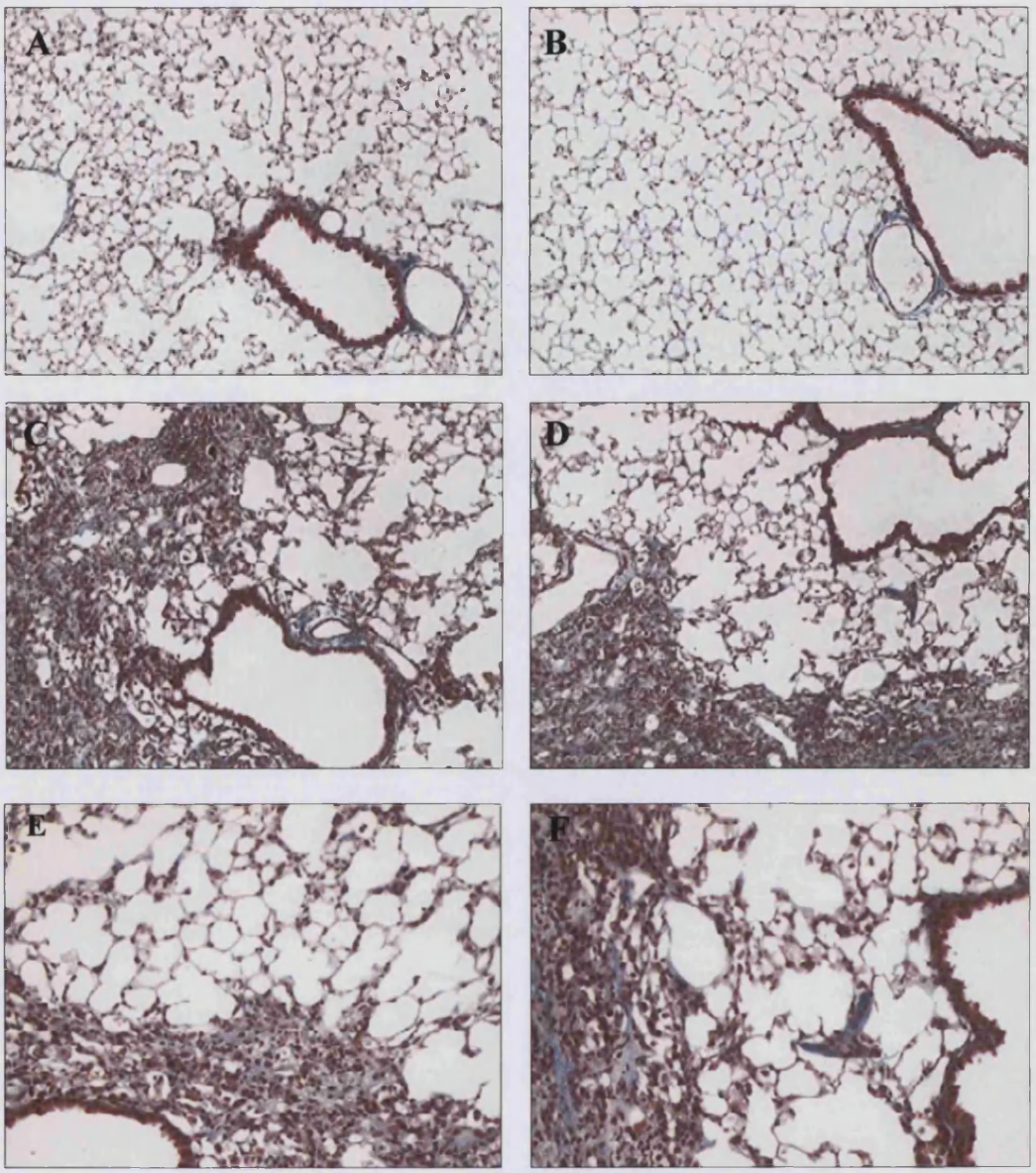


Figure 3.13. Effect of pharmacological inhibition of COX-2 using NS398 (3mg/kg) 28 days following IT instillation of either 0.9% saline or bleomycin (1mg/kg)

Micrographs of lung sections stained using modified MSB. A) vehicle treated and B) NS398-treated mice following instillation of saline alone. C) & E) vehicle-treated, D) & F) NS398-treated mice following instillation of bleomycin. Original magnification x100 (A-D) and x200 (E & F).

lead to an enhanced fibrotic response to bleomycin. However, despite almost total inhibition of PGE_2 production by NS398 for at least 14 days following injury, collagen production in the lungs of these mice was not further enhanced at either timepoint examined. The inflammatory response to bleomycin assessed by lavage cell profiles was also unaltered in mice treated with NS398 compared with vehicle alone.

The incomplete inhibition of PGE_2 production by day 28 following injury may confound the approach of pharmacological inhibition of COX-2 in this model. Therefore, an alternative strategy to inhibit COX-2 production, and therefore presumably induced PGE_2 synthesis following lung injury, would be to use gene disrupted animals. The use of COX-2 deficient mice in the bleomycin model of pulmonary fibrosis will be discussed in the following section.

Section 3 COX-2 deficient mice

Previous reports have shown COX-2 homozygous deficient ($COX\text{-}2^{-/-}$) mice to be unable to upregulate PGE_2 synthesis following a variety of injurious stimuli in a range of organs (Morteau *et al.*, 2000; Zeldin *et al.*, 2001; Bonner *et al.*, 2002). Therefore, it was hypothesised that following bleomycin injury $COX\text{-}2^{-/-}$ mice would show limited induction of PGE_2 synthesis, which in turn may result in a more severe fibrotic response to bleomycin-induced lung injury for reasons previously described.

COX-2 heterozygous deficient ($COX\text{-}2^{+/-}$) mice were also used. These animals have previously been shown to produce intermediate amounts of COX-2 at the RNA level (Dinchuk *et al.*, 1995). In addition, **Figure 3.14** shows intermediate production of COX-2 protein in the lung, both basally and following instillation of bleomycin compared with WT mice. As expected, $COX\text{-}2^{-/-}$ mice produce no COX-2 protein. Production of PGE_2 following lung injury has not previously been investigated in COX-2 heterozygous deficient mice.

3.11 Effect of COX-2 deficiency on body weight and weight changes following bleomycin instillation

All experiments in ***Section 3*** use littermate wild type mice as control animals. **Figure 3.15A** shows the mean body weights of WT, $COX\text{-}2^{+/-}$ and $COX\text{-}2^{-/-}$ mice aged 6-10

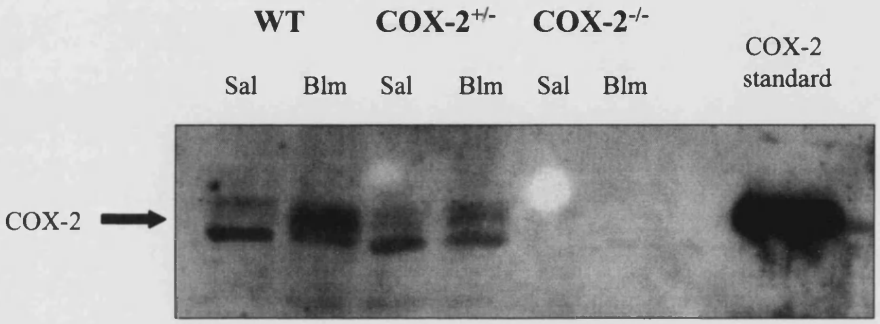


Figure 3.14 COX-2 expression in the lungs of WT, COX-2$^{+/-}$ and COX-2$^{-/-}$ mice following instillation of either saline or bleomcyin
Protein extracted from WT, COX-2$^{+/-}$ and COX-2$^{-/-}$ lungs was electrophoresed on SDS-PAGE gel and detected immunologically using a rabbit anti-mousenpolyclonal COX-2 antibody. Figure 3.14 is representative of a further 3 blots.

weeks before the start of all subsequent experiments. COX-2 deficiency has no effect on mean body weight compared with WT animals.

Following instillation of saline alone, all three genotypes showed little change in body weight for the first 7 days, **Figure 3.15B**. By day 14 there was a 1-2g increase in body weight in all three genotypes and at day 28 both WT and COX-$2^{-/-}$ mice gained 2.5-3g, significantly increased compared with day 7, $p<0.01$. Following instillation of bleomycin all three genotypes lost weight by day 3 but this was regained, and subsequently increased by day 14 **Figure 3.15C**. By day 28 all three genotypes gained 2-3.5g in weight although this was not significantly different to that observed following administration of saline alone in any genotype.

3.12 Measurement of BALF PGE_2 in COX-$2^{-/-}$ and COX-$2^{+/-}$ mice following intratracheal instillation of bleomycin

As previously shown in **Figures 3.3, 3.5** and **3.9**, lavage PGE_2 levels were significantly increased in WT mice for at least 28 days following intratracheal instillation of bleomycin. It was hypothesised that COX-$2^{-/-}$ mice would be unable to upregulate synthesis of PGE_2, however unexpectedly, **Figure 3.16** shows that this was not the case.

Control levels of BALF PGE_2 following administration of saline alone were low but measurable at each timepoint examined. Generally, BALF PGE_2 levels following instillation of saline were unaffected by either COX-2 genotype or time following instillation, however at day 14 BALF PGE_2 was significantly greater in WT mice compared with COX-$2^{+/-}$ animals, and this value is increased compared with WT BALF PGE_2 at day 7, $p<0.05$ in both cases. However BALF PGE_2 levels in COX-$2^{+/-}$ and COX-$2^{-/-}$ mice are not significantly different, either throughout the timecourse, or compared with WT or COX-$2^{-/-}$/COX-$2^{+/-}$ mice at any time.

Three days following administration of bleomycin all three genotypes showed an increase in BALF PGE_2 compared with instillation of saline alone, however this was only significant in COX-$2^{+/-}$ and COX-$2^{-/-}$ mice at this time ($p<0.05$). By 7 days following instillation of bleomycin, wild type mice significantly upregulated BALF PGE_2 production compared with instillation of saline alone, $p<0.05$ and levels were maintained at day 14. By day 28 following injury, levels of BALF PGE_2 had begun to

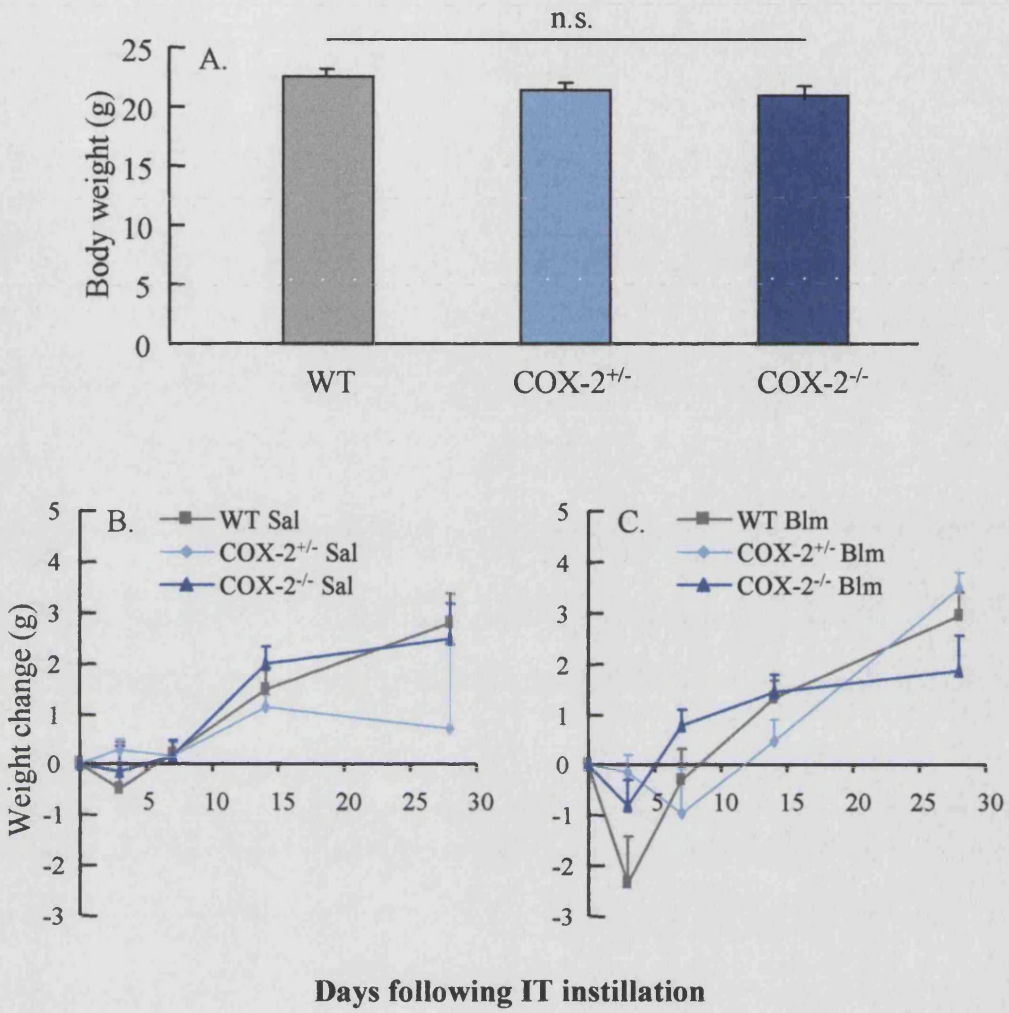


Figure 3.15. Effect of COX-2 genotype on body weight and weight change following instillation of saline or bleomycin

Figure 3.15A shows average starting body weight in grams of littermate B196 wild type mice compared with heterozygous and homozygous COX-2 deficient mice at 6-10 weeks of age, n=25. Weight change following IT administration of 0.9% saline is shown in Figure 3.15B and bleomycin (1mg/kg) in Figure 3.15C. At least 7-10 animals were used in each group in Figures 3.15B and C.

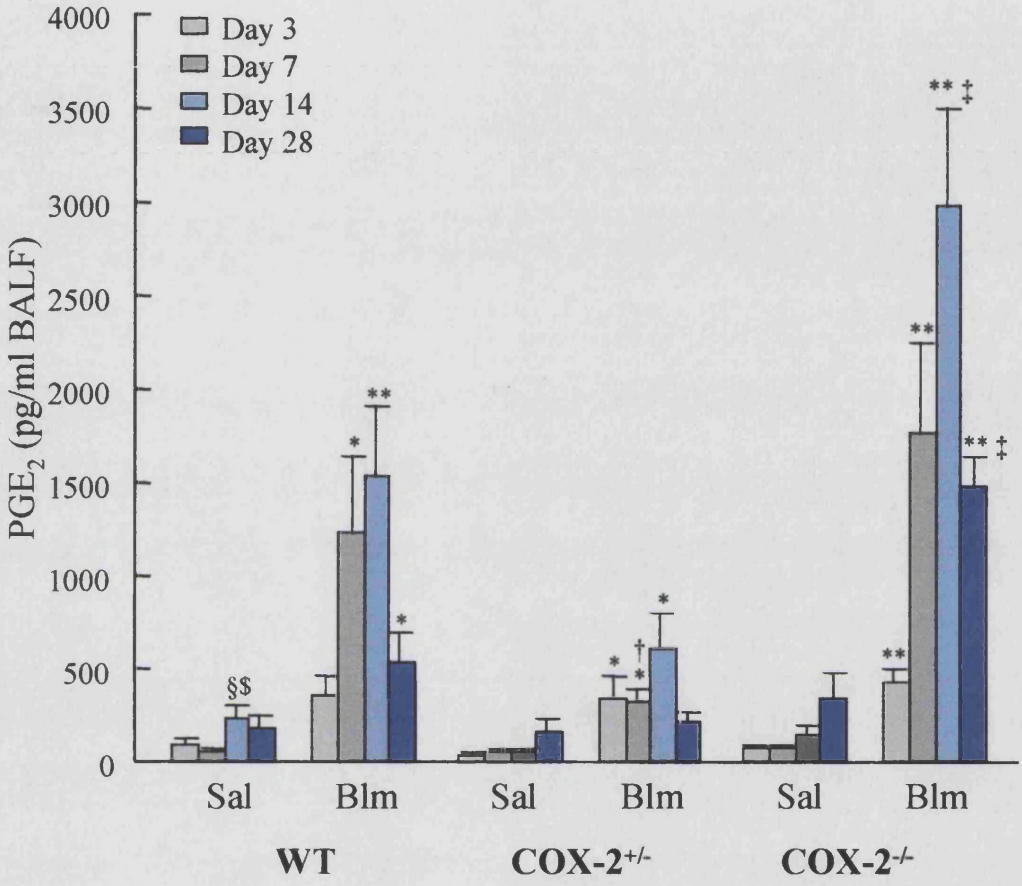


Figure 3.16. BALF PGE_2 levels in WT, COX-$2^{+/-}$ and COX-$2^{-/-}$ mice
Lungs were lavaged with PBS 3, 7, 14 or 28 days following IT administration of either bleomycin (1mg/kg) or saline. PGE_2 was measured using an EIA. Each bar represents the mean ± SEM of 7-9 mice. § represents $p<0.05$ compared with WT saline value at day 7, $ represents $p<0.05$ compared with COX-$2^{+/-}$ saline at day 14, * represents $p<0.05$ compared with respective genotype saline, ** represents $p<0.01$ compared with respective genotype saline, † represents $p<0.05$ compared with WT and COX-$2^{-/-}$ bleomycin at day 7, ‡ represents $p<0.05$ compared with WT and COX-$2^{+/-}$ mice bleomycin at day 14 and 28.

decrease, but were still significantly increased compared with WT mice that received saline alone, (Blm: 535 vs Sal: 177pg/ml BALF, $p<0.05$).

However, COX-$2^{-/-}$ mice showed compensatory biosynthesis of PGE_2 with significant upregulation of BALF PGE_2 following administration of bleomycin compared with saline alone at all timepoints examined, $p<0.001$. BALF PGE_2 production increased in a time-dependent manner at days 3, 7 and 14 and was maximal at day 14 where there was a 21-fold increase in BALF PGE_2 compared with saline alone. By day 28 levels had decreased compared with day 14, but remained significantly increased compared with instillation of saline alone.

At day 3, bleomycin-induced BALF PGE_2 levels in COX-$2^{-/-}$ mice were comparable with those of WT and COX-$2^{+/-}$ mice. However, by day 7 BALF PGE_2 production was significantly greater in COX-$2^{-/-}$ mice than COX-$2^{+/-}$ mice, $p<0.05$, with a trend towards an increase compared with WT animals. At both 14 and 28 days this trend became significant and BALF PGE_2 production in COX-$2^{-/-}$ mice was significantly greater than both COX-$2^{+/-}$ and WT animals at these times, $p<0.05$ in each case.

Mice heterozygous deficient for COX-2 showed no compensatory synthesis of PGE_2. Indeed, reduced expression of COX-2 in these animals (see **Figure 3.14**) resulted in a limited induction of PGE_2 synthesis in response to bleomycin. BALF PGE_2 production was significantly increased following bleomycin injury at days 3, 7 and 14 compared with saline alone, $p<0.05$, and peaked at day 14, similar to both WT and COX-$2^{-/-}$ mice. However, PGE_2 levels had declined by day 28 following instillation of bleomycin and were not significantly increased compared with instillation of saline alone, in contrast to both WT and COX-$2^{-/-}$ animals. Seven days following injury BALF PGE_2 was significantly lower in COX-$2^{+/-}$ mice than either WT or COX-$2^{-/-}$ animals exposed to bleomycin ($p<0.05$) with a trend towards decreased levels of PGE_2 at both days 14 and 28.

Together these data suggest a persistent compensatory upregulation of PGE_2 in COX-$2^{-/-}$ mice following injury and a reduced capacity to upregulate PGE_2 synthesis in mice heterozygous for COX-2.

3.13 Measurement of total lung collagen in COX-2$^{-/-}$ and COX-2$^{+/-}$ mice following bleomycin injury

The murine bleomycin model of pulmonary fibrosis is characterised by a significant increase in total lung collagen by day 14. By using a relatively low dose (1mg/kg) of bleomycin it was hoped an enhanced fibrotic response to bleomycin-induced lung injury would be observed in COX-2 deficient animals compared with wild type controls. The fibrotic response to bleomycin was evaluated in two ways, firstly biochemically by measuring hydroxyproline content of the lungs using reverse-phase HPLC, and secondly histologically using sections stained with the matrix stain MSB.

Figure 3.17 shows that COX-2 does not affect basal collagen production as total lung collagen is similar in all three genotypes 14 days following instillation of bleomycin. Following bleomycin injury, WT mice show the characteristic increase in lung collagen production as previously shown in **Figure 3.4**. Bleomycin also causes increased collagen production in both COX-2$^{+/-}$ and COX-2$^{-/-}$ mice at this time, $p<0.05$. The increase tended to be greater in COX-2$^{+/-}$ mice although this was not significantly greater than either WT or COX-2$^{-/-}$ mice.

At the second timepoint examined, basal collagen levels were also unaffected by COX-2 genotype (**Figure 3.18**). Total lung collagen content 28 days following instillation of saline was neither different between WT or COX-2 deficient ($^{+/-}$ or $^{-/-}$) mice , nor was it different compared with comparable basal levels at day 14 (**Figure 3.17**).

Again, instillation of bleomycin caused a significant increase in total lung collagen content 28 days following injury in all three genotypes compared with instillation of saline alone ($p<0.01$ WT mice, $p<0.001$ COX-2$^{+/-}$ mice, $p<0.05$ COX-2$^{-/-}$ mice, **Figure 3.18**). Both WT and COX-2$^{-/-}$ mice showed a trend towards a time-dependent increase in lung collagen content at day 28 compared with day 14 (**Figure 3.17**), although this increase did not reach statistical significance. However, COX-2$^{+/-}$ mice showed a highly significant time-dependent increase in lung collagen production; by day 28 collagen content had increased over 50% compared with day 14, (14 days 2.48mg vs 28 days 3.77mg, $p<0.001$). Furthermore, total lung collagen in COX-2$^{+/-}$ mice at day 28 was approximately 53% greater than that observed in WT mice and 50% greater than COX-2$^{-/-}$ mice, $p<0.001$ in both cases.

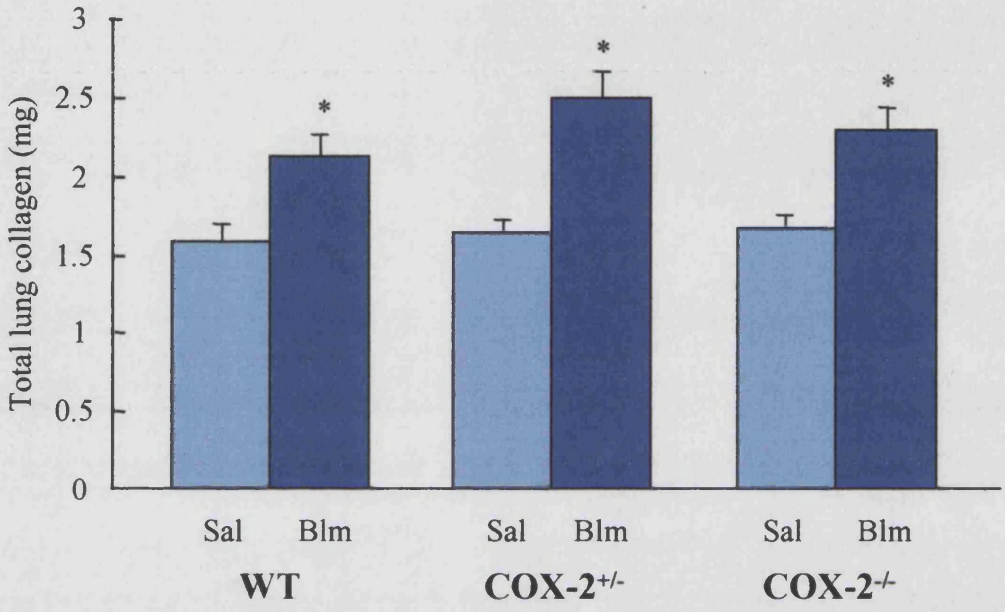


Figure 3.17. Effect of saline and bleomycin on lung collagen content 14 days following administration

Saline (0.9%) or bleomycin (1mg/g) were instilled into the lung. After 14 days the lungs were harvested and hydroxyproline measured using HPLC. Each value represents the mean ± SEM for 8-10 animals. * indicates $p<0.05$ compared with genotype saline control.

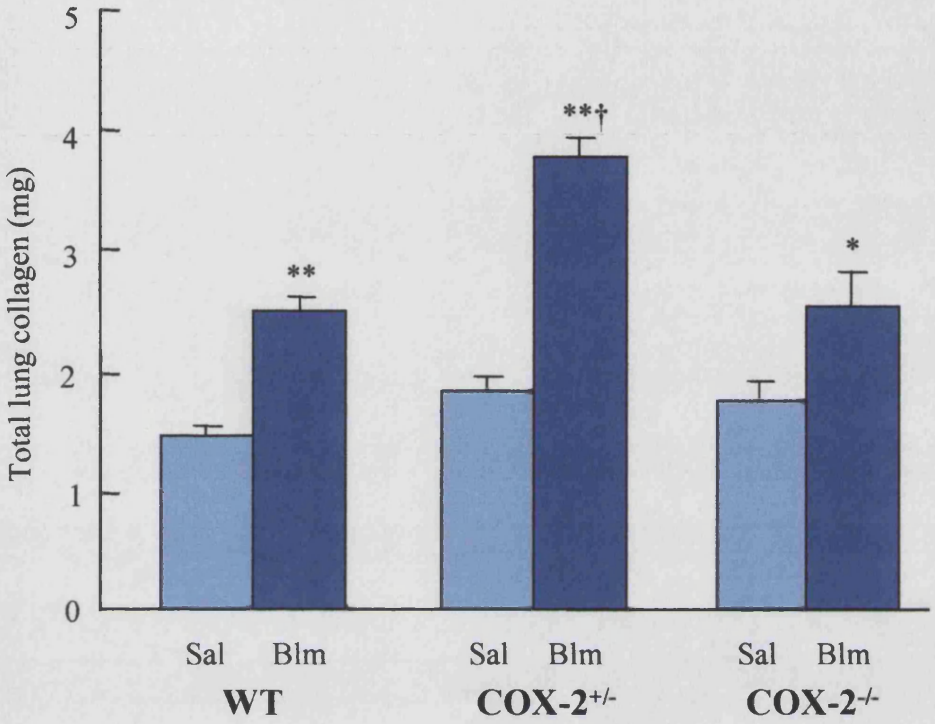


Figure 3.18. Effect of saline and bleomycin on lung collagen content 28 days following administration.
Saline (0.9%) or bleomycin (1mg/kg) were instilled into the lung. After 28 days the lungs were harvested and hydroxyproline measured using HPLC. Each value represents the mean ± SEM for 8-13 animals. * indicates $p<0.05$ & ** indicates $p<0.01$ compared with genotype saline control. † indicates $p<0.001$ compared with COX-$2^{+/+}$ & COX-$2^{-/-}$ blm.

3.14 Histological analysis of bleomycin induced fibrosis

Histological analysis of lung sections stained using MSB show collagen stained blue whilst inflammatory cells appear red. Similarly to measurements of total lung collagen, histological analysis 28 days following bleomycin showed greater staining for matrix proteins in COX-$2^{+/-}$ mice compared with both WT and COX-$2^{-/-}$ mice. **Figure 3.19A-C** shows all three COX-2 genotypes 28 days following instillation of saline alone. Normal lung architecture was retained, with thin alveolar walls and matrix staining limited to blood vessels, airways and normal alveolar interstitium. No apparent differences were seen between genotypes.

In contrast **Figure 3.20A-E** shows the lungs 28 days following intratracheal instillation of bleomycin. The intensity of the blue stain was markedly increased compared with saline treated mice in all genotypes. Both WT and COX-$2^{-/-}$ mice showed a patchy loss of alveolar architecture and destruction of the interstitium, accompanied by increased matrix deposition in areas where alveolar architecture was disrupted. In COX-$2^{+/-}$ mice, bleomycin injury caused an aggressive fibrotic response resulting in widespread destruction of lung architecture which was more severe and more extensive than that seen in either WT or COX-$2^{-/-}$ mice (**Figures 3.20A, B, D**).

Higher power shows that affected areas of the lung in COX-$2^{+/-}$ mice were associated with intense staining for matrix proteins, and increased cellularity (**Figure 3.20C**). This histological data correlates with biochemical measurements of total lung collagen production at day 28 in these animals (**Figure 3.18**). The lesions in COX-$2^{-/-}$ mice contained reduced amounts of matrix staining but an increased mixed inflammatory response.

3.15 Effect of COX-2 genotype on inflammatory response to bleomycin

To determine the effect of COX-2 genotype on the inflammatory response, total cell numbers were measured, and the relative proportions of inflammatory cells were assessed by differential Diff-Quick staining of cytospins from BALF obtained at various timepoints following administration of either bleomycin or saline.

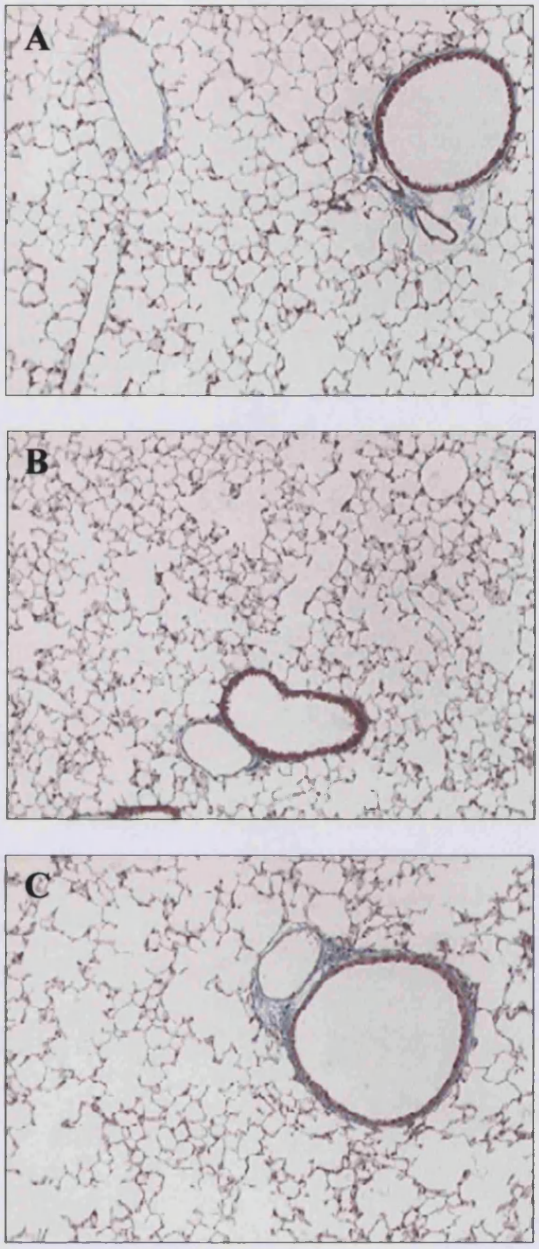


Figure 3.19. Effect of intratracheal instillation of 0.9% saline on the lungs of WT, COX-2$^{+/-}$ and COX-2$^{-/-}$ mice

Low power micrographs of lung sections stained using modified MSB from A) WT, B) COX-2$^{+/-}$ and C) COX-2$^{-/-}$ mice 28 days following instillation of saline. Original magnification x100.

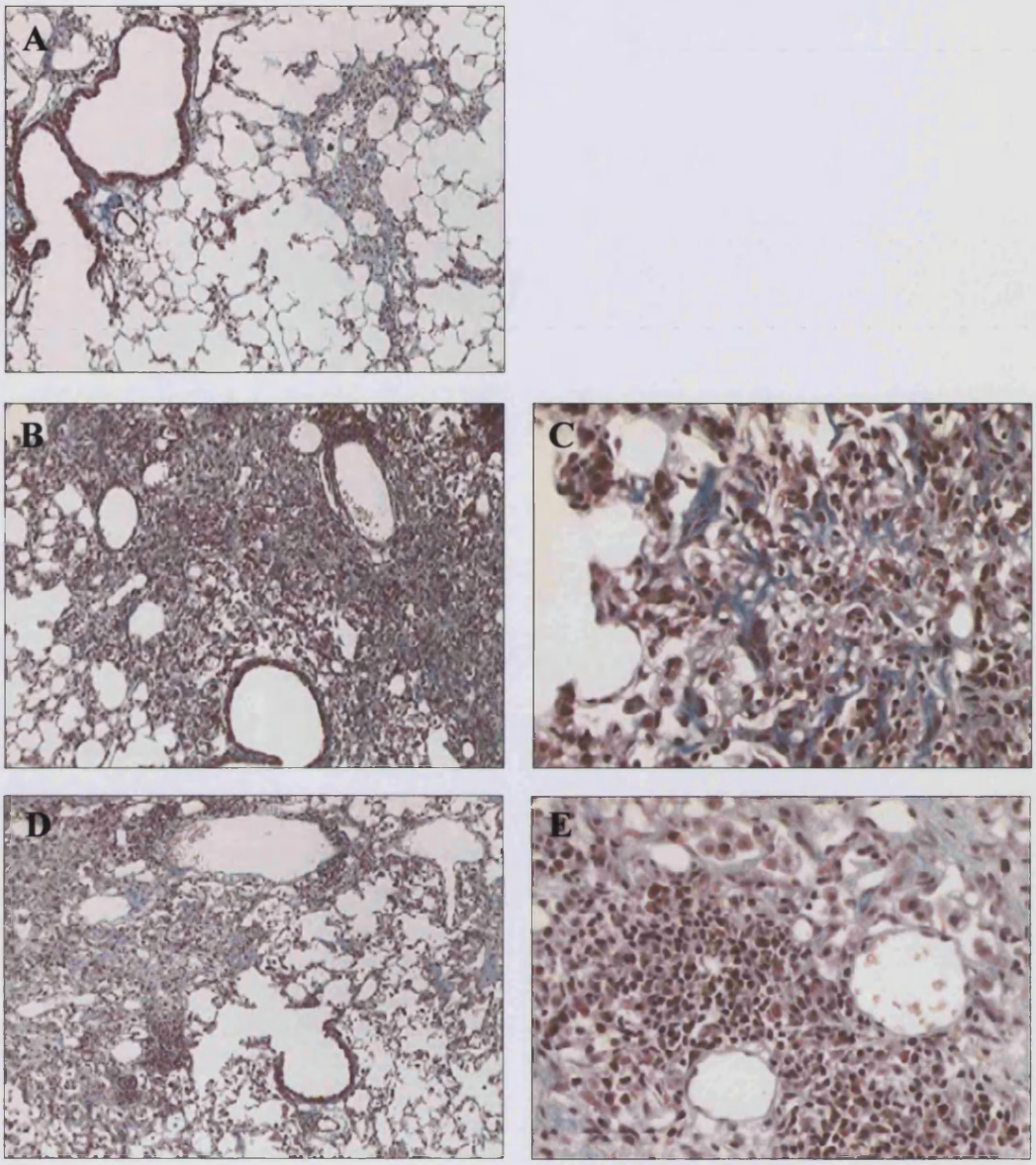


Figure 3.20. Effect of intratracheal instillation of 1mg/kg bleomycin on the lungs of WT, COX-$2^{+/-}$ and COX-$2^{-/-}$ mice

Micrographs of lung sections stained using modified MSB from A) WT, B) & C) COX-$2^{+/-}$ and D) & E) COX-$2^{-/-}$ mice 28 days following instillation of bleomycin. Original magnification x100 (A, B, D) and x400 (C & E).

Total BALF cell numbers from animals instilled with saline were not significantly different between genotypes or at any timepoint (**Table 3.2**) Three days following bleomycin instillation total BAL cell numbers tended to be lower than for animals instilled with saline but these differences were not statistically significant. Between 7 and 28 days following bleomycin BAL cell numbers tended to be increased by 30-200% although due to variability between animals these differences were not always significant (**Table 3.2**). In WT mice total cell numbers increased following bleomycin injury at days 7, 14 and 28 compared with respective saline control animals. BAL cell numbers were increased in COX-$2^{+/-}$ mice at days 14 and 28 following injury, and a significant increase in total BAL cell numbers was seen in COX-$2^{-/-}$ mice at 28 days alone, $p<0.05$ in all cases. There was no clear difference in BAL total cell numbers between genotypes at any timepoint, although the highest cell numbers was observed in COX-$2^{-/-}$ animals 28 days following bleomycin, with value three times that of saline treated animals.

BALF cell profiles were analysed at 3, 7, 14 and 28 days following injury and did not alter significantly between genotypes in response to saline alone. In WT animals macrophage numbers increased following bleomycin and became significantly greater than saline alone 14 days following injury (Sal 163 ±23.7; Blm 387.5 ±44.9 $x10^{-3}$ cells, $p<0.05$, **Table 3.2.**). Macrophage numbers in COX-$2^{+/-}$ and COX-$2^{-/-}$ animals were significantly increased following bleomycin at 28 days (COX-$2^{+/-}$ Sal 252 ±70.5; Blm 485.6 ±60.9; COX-$2^{-/-}$ Sal 169 ±30.2 Blm 410.1 ±69.5, $p<0.05$ in both cases), however no differences in total BAL macrophage number were seen between genotypes at any timepoint examined (**Table 3.2**).

In contrast COX-2 genotype did affect polymorphonuclear leukocyte (PMN) and lymphocyte numbers following bleomycin administration. At days 3, 7 and 14 numbers of PMNs increased in all genotypes following instillation of bleomycin, peaking at day 7. Although again, due to variability between animals these differences were not always significant (**Table 3.2**). Significant increases were seen at day 7 in both WT and COX-$2^{-/-}$ mice, $p<0.05$, and at day 14 in COX-$2^{+/-}$ and COX-$2^{-/-}$ mice, $p<0.05$. By day 28 numbers of PMNs decreased in WT mice, and were no longer significantly elevated over saline alone in both WT and COX-$2^{+/-}$ mice, however numbers of BAL PMNs remained elevated in COX-$2^{-/-}$ mice. At 28 days following administration of bleomycin, BAL PMN numbers were significantly greater in COX-$2^{-/-}$ mice compared with both

Day	Genotype	Treatment	Inflammatory cell number ($x10^{-3}$)			
			Total cells	Macrophage	PMN	Lymphocyte
3	WT	Saline	238.5 ± 34.0	237.2 ± 3.8	1.1 ±0.4	0.4 ±0.2
	WT	Bleomycin	195.0 ± 31.6	168.5 ±28.7	24.2 ±19.1	1.7 ±0.2 *
	COX-$2^{+/-}$	Saline	310.6 ± 35.2	298.9 ±35.3	10.3 ±5.0	1.3 ±0.7
	COX-$2^{+/-}$	Bleomycin	229.7 ± 43.2	188.1 ±30.5	38.6 ±26.2	3.0 ±0.9
	COX-$2^{-/-}$	Saline	254.5 ± 32.9	252.2 ±32.5	1.2 ±0.4	1.0 ±0.4
	COX-$2^{-/-}$	Bleomycin	231.9 ± 20.3	193.3 ±10.2	35.6 ±19	3.3 ±1.3
7	WT	Saline	346.0 ± 38.5	335.0 ±39.0	6.0 ±4.0	5.2 ±3.6
	WT	Bleomycin	459.3 ± 59.2 *	423.0 ±53.7	61.0 ±14.7 *	28.2 ±6.9 *
	COX-$2^{+/-}$	Saline	277.4 ± 27.0	270.5 ±26.4	3.3 ±0.7	3.5 ±1.8
	COX-$2^{+/-}$	Bleomycin	407.7 ± 53.0	313.8 ±43.0	70.9 ±34.9	22.3 ±10.3
	COX-$2^{-/-}$	Saline	361.4 ± 63.7	352.8 ±59.1	4.6 ±2.3	4.0 ±2.8
	COX-$2^{-/-}$	Bleomycin	493.2 ± 52.5	310.7 ±45.6	86.2 ±22.4 *	66.3 ±22.4 *
14	WT	Saline	177.9 ± 27.7	163.4 ±23.7	6.5 ±5.4	8.2 ±3.5
	WT	Bleomycin	430.4 ± 50.9 *	387.5 ±44.9 *	25.8 ±10.0	17.9 ±2.3 *
	COX-$2^{+/-}$	Saline	230.0 ± 40.9	242.2 ±51.5	1.5 ±0.3	0.6 ±0.6
	COX-$2^{+/-}$	Bleomycin	428.3 ± 40.1 *	403.0 ±58.3	11.6 ±2.2 *	16.7 ±7.3
	COX-$2^{-/-}$	Saline	207.3 ± 33.1	207.3 ±33.1	1.5 ±0.4	0.4 ±0.3
	COX-$2^{-/-}$	Bleomycin	319.9 ± 51.7	327.3 ±55.4	23.0 ±5.8 *	23.6 ±7.4 *
28	WT	Saline	207.7 ± 21.6	202.0 ±21.4	1.7 ±0.9	3.6 ±1.2
	WT	Bleomycin	357.6 ± 39.6 *	340.7 ±38.0 *	9.3 ±3.3	7.7 ±2.5
	COX-$2^{+/-}$	Saline	258.9 ± 71.6	252.0 ±70.5	4.0 ±1.0	2.6 ±0.8
	COX-$2^{+/-}$	Bleomycin	520.5 ± 68.1 *	485.6 ±60.9 *	27.1 ±13.3	7.7 ±1.9 *
	COX-$2^{-/-}$	Saline	174.2 ± 31.6	169.0 ±30.2	1.8 ±0.7	3.4 ±2.1
	COX-$2^{-/-}$	Bleomycin	534.0 ± 84.4 *	410.1 ±69.5 *	73.8 ±26.9 *†	50.7 ±16.2 *†

Table 3.2. Effect of COX-2 genotype on total inflammatory response
Animals were lavaged with PBS at 3, 7 14 and 28 days following IT instillation of either saline of bleomycin (1mg/kg). Total cell numbers were determined using a haemocytometer and expressed as mean ±SEM total cell count ($x10^{-3}$). Cytospin preparations were made to obtain differential cell counts and expressed as mean ±SEM cell number ($x10^{-3}$). 6-9 animals were used in each group. * indicates $p<0.05$ compared with relative genotype saline, † indicates $p<0.05$ compared with WT and COX-$2^{+/-}$ bleomycin at 28 days.

WT and COX-$2^{+/-}$ mice (**Table 3.2**, WT: 11.1 ±5.6; COX-$2^{+/-}$: 27.1 ±13.3; COX-$2^{-/-}$: 73.8 ±26.9, $p<0.05$).

Lymphocyte numbers followed a similar pattern (**Table 3.2**). In all three genotypes instillation of bleomycin caused an increase in numbers of BAL lymphocytes at all timepoints examined compared with saline alone. Again, due to variation between animals these differences were significant in WT mice at days 3, 7 and 14 days, in COX-$2^{+/-}$ mice at day 28 alone, and in COX-$2^{-/-}$ mice at days 7, 14 and 28, $p<0.05$ in all cases. By 28 days following bleomycin numbers of BAL lymphocytes were significantly greater in COX-$2^{-/-}$ mice compared with both WT and COX-$2^{-+/-}$ animals (WT: 7.7 ±2.5; COX-$2^{+/-}$ 7.7 ±1.9; COX-$2^{-/-}$ 50.7 ±16.2, $p<0.05$ in both cases).

3.16 Heterozygote COX-2 deficient mice as a progressive model of pulmonary fibrosis

Pulmonary fibrosis is a progressive process, however the progressive nature of this disease is a feature which has been shown to be difficult to reproduce in animal models following one injurious stimulus. To investigate if the enhanced fibrotic response resulting from decreased expression of COX-2 and reduced PGE_2 synthesis can result in a perpetuation of a progressive response, animals were left for an extended timecourse of 84 days. After this time the fibrotic response was analysed histologically and by the measurement of total lung collagen. Additionally, the effect of a longer timecourse on COX-2 homozygous deficient mice was examined.

3.16.1. PGE_2 production 84 days following injury

At 84 days following the IT administration of either 0.9% saline or bleomycin (1mg/kg), BALF PGE_2 levels remained measurable in all groups. However BALF PGE_2 was no longer significantly increased following bleomycin injury compared with administration of saline alone in any group. PGE_2 synthesis in homozygous COX-2 deficient mice was no longer increased compared with either WT or COX-$2^{+/-}$ mice. **Figure 3.21** shows the values in relation to those values at day 28.

3.16.2 Total lung collagen production 84 days following injury

Figure 3.22 represents total lung collagen content at day 84 compared with values observed at 28 days following either administration of saline or bleomycin (1mg/kg). At

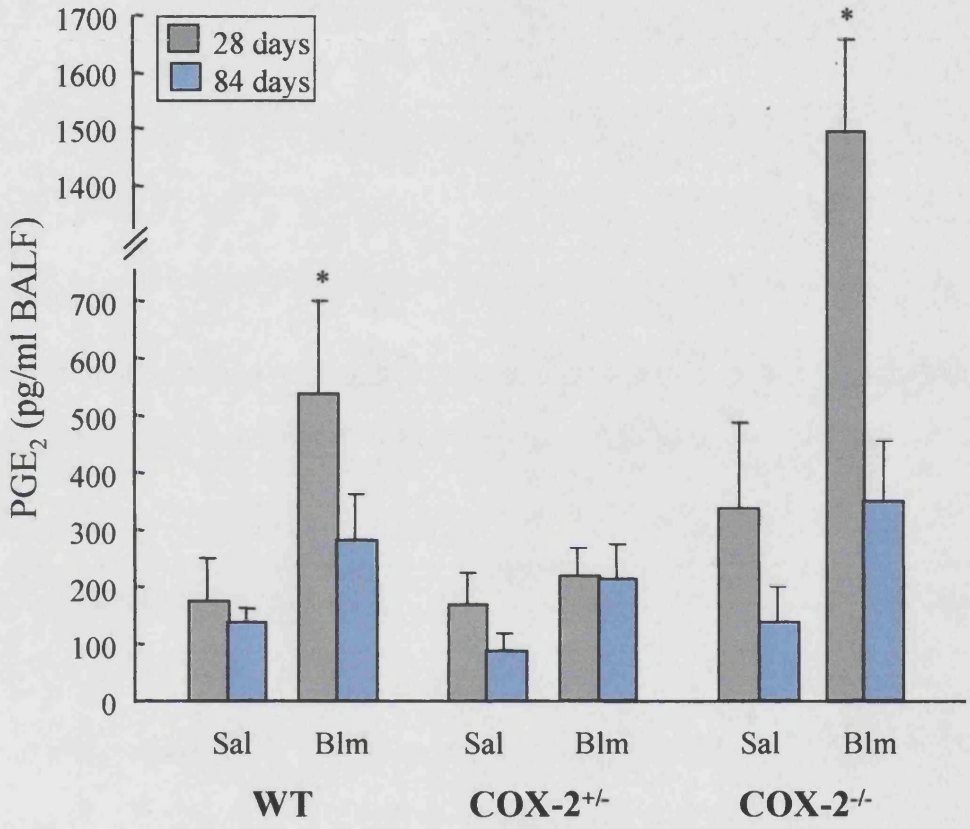


Figure 3.21. BALF PGE_2 28 and 84 days following intratracheal instillation of saline or bleomycin

Animals were lavaged with PBS at 28 and 84 days following instillation of bleomycin (1mg/kg). BALF PGE_2 was measured using a commercially available EIA. * indicates $p<0.05$ compared with saline control. 6-11 animals were used per group.

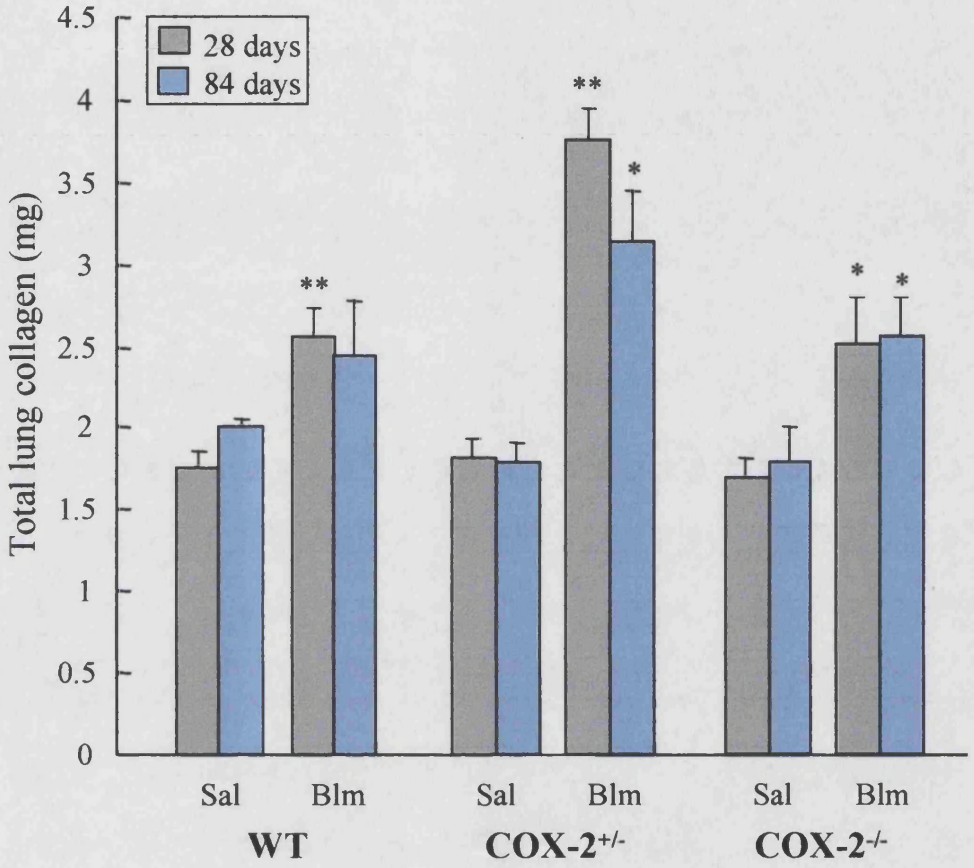


Figure 3.22. Total lung collagen 28 and 84 days following bleomycin-induced lung injury

Lungs were removed and total lung collagen measured using HPLC. Each bar represents the mean ±SEM for 6-12 animals per group. * indicates $p<0.05$; ** $p<0.01$ compared with genotype saline control.

this timepoint, levels of total lung collagen, either following administration of saline or bleomycin, were not significantly different compared with values at day 28 in any genotype. This suggests that the fibrotic response was maintained, but not enhanced at this time.

3.16.3 Histological assessment of lung injury 84 days following injury

Histological analysis of lung sections obtained 84 days following administration of either saline or bleomycin, shows similar features to those observed at the earlier timepoint of 28 days (**Figures 3.19** and **3.20**). **Figure 3.23A-C** shows all three COX-2 genotypes 84 days following instillation of saline alone. Normal lung architecture was still apparent, with thin alveolar walls and matrix staining limited to blood vessels, airways and normal alveolar interstitium, with no difference between genotypes.

Figure 3.24A-C shows the lungs 84 days following intratracheal instillation of bleomycin. Similarly to day 28, the intensity of the blue stain remained markedly increased compared with saline treated mice in all genotypes. Again, all three genotypes show patchy fibrotic lesions in response to bleomycin, with disruption of alveolar architecture, destruction of the interstitium and increased matrix deposition in affected areas. Mixed inflammatory cell infiltrates were seen in affected areas of all genotypes. The severe fibrotic response seen in COX-$2^{+/-}$ mice at day 28 appeared to be maintained at day 84. A greater number of affected areas were seen in COX-$2^{+/-}$ mice compared with either WT or COX-$2^{-/-}$ animals, typical lesions are shown in (**Figures 3.24A, B, C**). This histological data correlates with the biochemical measurements of total lung collagen production at day 84 (**Figure 3.22**).

3.16.4 Inflammatory response at day 84

By day 84 following administration of bleomycin, total cell numbers were not significantly different compared with administration of saline alone in WT or COX$^{-/-}$ mice, although a significant increase remained in COX-$2^{+/-}$ mice, $p<0.05$ (**Table 3.3**). Total cell numbers following the administration of saline alone were similar in all genotypes at day 84. Furthermore cell number following saline had not changed significantly in any genotype throughout the whole of the 84 day timecourse.

Numbers of PMNs were not increased following bleomycin injury in WT mice at day 84, however increases were still observed in both COX-$2^{+/-}$ and COX-$2^{-/-}$ mice, although

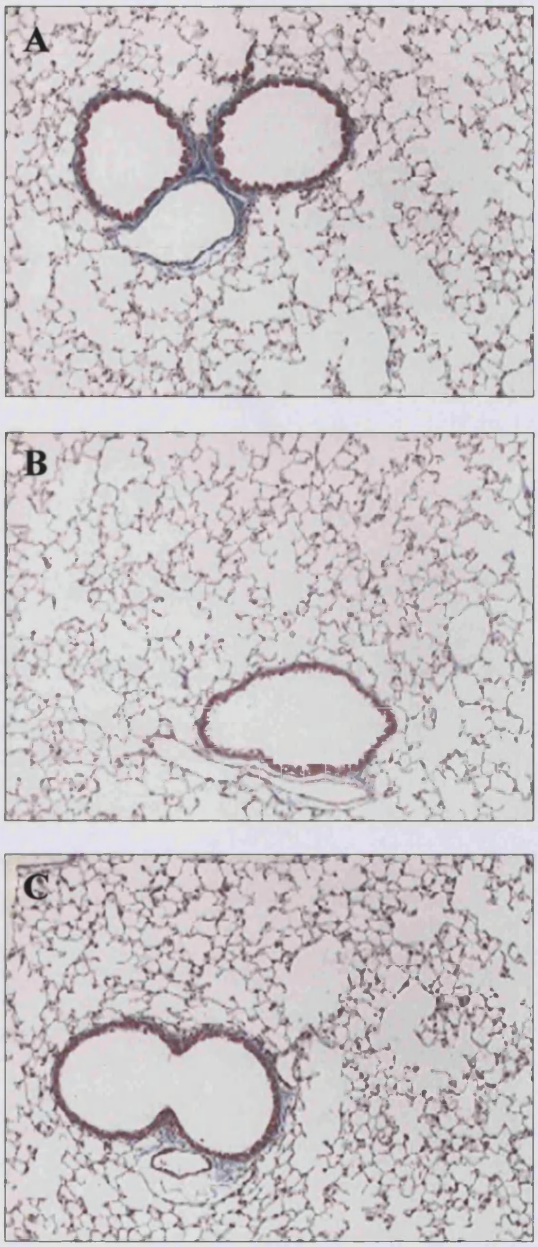


Figure 3.23. Effect of intratracheal instillation of 0.9% saline on the lungs of WT, COX-$2^{+/-}$ and COX-$2^{-/-}$ mice

Low power micrographs of lung sections stained using modified MSB from A) WT, B) COX-$2^{+/-}$ and C) COX-$2^{-/-}$ mice 84 days following instillation of saline. Original magnification x100.

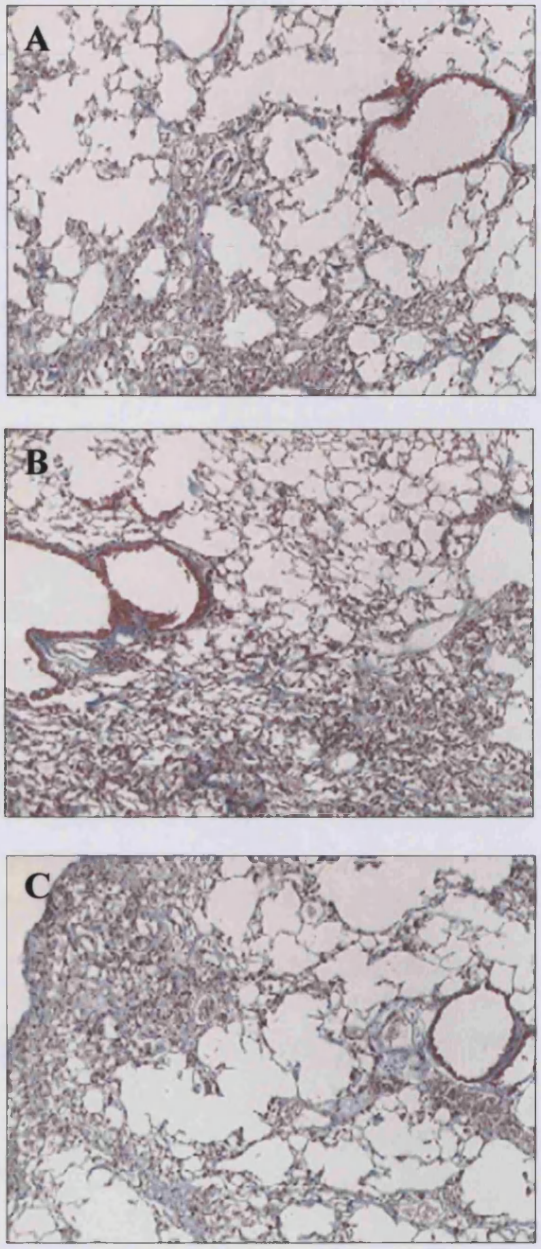


Figure 3.24. Effect of intratracheal instillation of 1mg/kg bleomycin on the lungs of WT, COX-$2^{+/-}$ and COX-$2^{-/-}$ mice
Low power micrographs of lung sections stained using modified MSB from A) WT, B) COX-$2^{+/-}$ and C) COX-$2^{-/-}$ mice 84 days following instillation of bleomycin. Original magnification x100.

Genotype	Treatment	Inflammatory cell number (x10^3)			
		Total cells	Macrophage	PMN	Lymphocyte
WT	Saline	184.7 ±18.1	176.1 ±20.4	2.4 ±1.0	8.3 ±2.8
WT	Bleomycin	245.7 ±28.8	232.0 ±23.9	1.4 ±0.7	12.3 ±7.0
COX-2$^{+/-}$	Saline	153.9 ±12.0	148.9 ±11.7	1.1 ±3.4	3.9 ±1.4
COX-2$^{+/-}$	Bleomycin	277.3 ±18.0 *	256.4 ±25.0 *	3.4 ±1.5	18.7 ±10.4
COX-2$^{-/-}$	Saline	205.5 ±41.7	174.8 ±27.2	1.8 ±1.7	12.4 ±7.2
COX-2$^{-/-}$	Bleomycin	243.4 ±18.4	191.1 ±14.4	6.0 ±1.7*†	46.4 ±14.0 *†

Table 3.3 Inflammatory cell numbers 84 days following IT administration of either saline or bleomycin

Animals were lavaged with PBS 84 days following administration of either 0.9% saline or 1mg/kg bleomycin. Total cell numbers were determined using a haemocytometer and expressed as mean ±SEM total cell count (x10^{-3}). Cytospin preparations were made to obtain differential cell counts and expressed as mean ±SEM cell number (x10^{-3}). 7-10 animals were used in each group. * indicates $p<0.05$ compared with relative genotype saline, † indicates $p<0.05$ compared with WT bleomycin.

this was only significant in COX-$2^{-/-}$ animals (**Table 3.3**, $p<0.05$). Numbers of BAL PMNs in COX-$2^{-/-}$ mice also remained significantly greater than those seen in WT mice, similarly to day 28, $p<0.05$, although not COX-$2^{+/-}$ mice by day 84.

A similar pattern was seen in BAL lymphocyte numbers; only COX-$2^{-/-}$ mice showed increased numbers of lymphocytes following instillation of bleomycin compared with saline alone at day 84, $p<0.05$. Again, BAL lymphocyte numbers in COX-$2^{-/-}$ mice were significantly greater than those seen in WT mice, but not COX-$2^{+/-}$ mice at this time (**Table 3.3**, WT: 12.3 ±7; COX-$2^{+/-}$: 18.7 ±10.4; COX-$2^{-/-}$: 46.4 ±14, $p<0.05$ compared with WT mice).

3.17 Summary: Effect of COX-2 genotype on the development of bleomycin-induced lung fibrosis

It has been shown that both COX-$2^{+/-}$ and COX-$2^{-/-}$ mice have phenotypes distinctly different in many aspects from WT control animals. COX-2 deficiency does not affect the body weight of these animals, nor does it affect the basal BALF PGE_2 production or total lung collagen content following instillation of saline alone. However, following instillation of bleomycin, differences exist in the response to bleomycin induced lung injury, the inflammatory response and the development of fibrosis.

Surprisingly, total absence of functional COX-2 protein (**Figure 3.7**) in COX-$2^{-/-}$ mice did not limit the upregulation of BALF PGE_2 synthesis (**Figure 3.16**) which was previously shown to be predominantly COX-2-derived (**Figure 3.9**). These animals did not show a more severe fibrotic response to bleomycin, as hypothesised (**Figures 3.17, 3.18** and **3.22**). However COX-$2^{-/-}$ mice did show an enhanced inflammatory response to bleomycin injury, both at days 28 and 84 (**Tables 3.2** and **3.3**). Experiments to identify potential explanations for this enhanced inflammatory response, together with a possible explanation for the compensatory synthesis of PGE_2 in these mice will be presented in ***Section 4***.

Mice heterozygous for COX-2 showed reduced protein expression in the lung (**Figure 3.7**) and following bleomycin challenge, this reduction in COX-2 expression resulted in only limited upregulation of BALF PGE_2 synthesis (**Figure 3.16**). With reduced levels of the antifibrotic PGE_2 present in the lung COX-$2^{+/-}$ mice demonstrate a more severe

fibrotic response to bleomycin compared with both WT and COX-$2^{-/-}$ animals. Total lung collagen accumulation was 53% greater than that seen in WT mice, and 50% greater than COX-$2^{-/-}$ mice animals (**Figure 3.18**). At the longer timepoint of 84 days following injury this difference was maintained, although not enhanced (**Figure 3.22**) and therefore COX-$2^{+/-}$ mice do not represent a progressive model of pulmonary fibrosis over an 84 day timepoint with only one initial instillation of bleomycin. The enhanced fibrotic response seen in COX-$2^{+/-}$ mice was not associated with a more severe inflammatory response to bleomycin as the inflammatory response was generally unchanged compared with WT mice at each timepoint examined and less severe than COX-$2^{-/-}$ mice (**Tables 3.2** and **3.3**).

Section 4 Compensatory mechanisms in COX-2 homozygous deficient mice

The characterisation of the COX-$2^{-/-}$ mouse phenotype in response to bleomycin describes a compensatory upregulation in PGE_2 synthesis following injury, however this phenomena is not observed in the absence of an injury, nor does it occur in mice heterozygous for the gene. This unexpected upregulation of BALF PGE_2 following instillation of bleomycin is accompanied by an enhanced and persistent inflammatory response characterised by increased numbers of PMNs and lymphocytes, but this is not accompanied by an enhanced fibrotic response to the injury, which is observed in mice heterozygous for COX-2. The following section aims to further investigate the mechanism and source of the increased PGE_2 seen in COX-$2^{-/-}$ mice and to investigate possible reasons for the enhanced inflammatory response following injury.

Firstly, western blotting was used to determine if increased PGE_2 production can be explained either by an increase in substrate availability, via an increase in cytosolic phospholipase A_2 ($cPLA_2$) production, or alternatively, due to a compensatory upregulation of COX-1.

3.18 Expression of $cPLA_2$ in lung tissue following injury

PGE_2 production has been shown to peak at approximately day 14, therefore, whole lung tissue was removed at this time and powdered under liquid nitrogen. Protein was

extracted by repeat freeze-thaw cycles and electrophoresed through SDS-PAGE gels. This was then transferred onto nitrocellulose membranes and probed for $cPLA_2$ using a specific antibody. These blots, and those described in **section 3.19** were predominantly performed by **Dr Caroline Wheeler-Jones** at the Royal Veterinary College, London.

Figure 3.25 shows that $cPLA_2$, the rate limiting enzyme in the release of arachidonic acid, is expressed at similar levels in the lungs of both saline treated WT and COX-$2^{-/-}$ mice 14 days following IT instillation. However, following bleomycin injury, $cPLA_2$ was upregulated approximately 1.5-fold in both WT and COX-$2^{-/-}$ lung tissue, but no difference was observed between the COX genotypes. Therefore these data suggest that the altered production of PGE_2 in COX-$2^{-/-}$ mice can not be explained by an increase in substrate availability due to increased $cPLA_2$ expression.

3.19 Expression of COX-1 in lung tissue following injury

Since COX-2 deficiency did not effect expression of cPLA2, levels of COX-1 were assessed. Again, protein extracted from whole lung tissue removed 14 days following administration of either saline or bleomycin, was electrophoresed through SDS-PAGE gels and transferred onto nitrocellulose membranes. The membranes were then probed for COX-1 using a specific antibody.

Figure 3.26 shows the expression of COX-1 protein in WT and COX-$2^{-/-}$ mice. Following the instillation of saline alone, COX-1 expression was similar in the lungs of both WT and COX-$2^{-/-}$ mice. However, following intratracheal instillation of bleomycin COX-1 protein expression was upregulated approximately 4.5 fold in COX-$2^{-/-}$ mice compared with only a 1.5-fold increase in wild type control animals. These data suggest that upregulation of PGE_2 in the lungs of COX-$2^{-/-}$ mice is, at least in part, mediated via a compensatory upregulation in COX-1 production, and not via an increase in $cPLA_2$ leading to increased arachidonic acid availability.

3.20 Compensatory upregulation of PGE_2 in vitro

Both fibroblasts and macrophages are cell types known to produce significant levels of PGE_2 in the lung and therefore the capacity of these COX-2 deficient cells to synthesise PGE_2 was investigated.

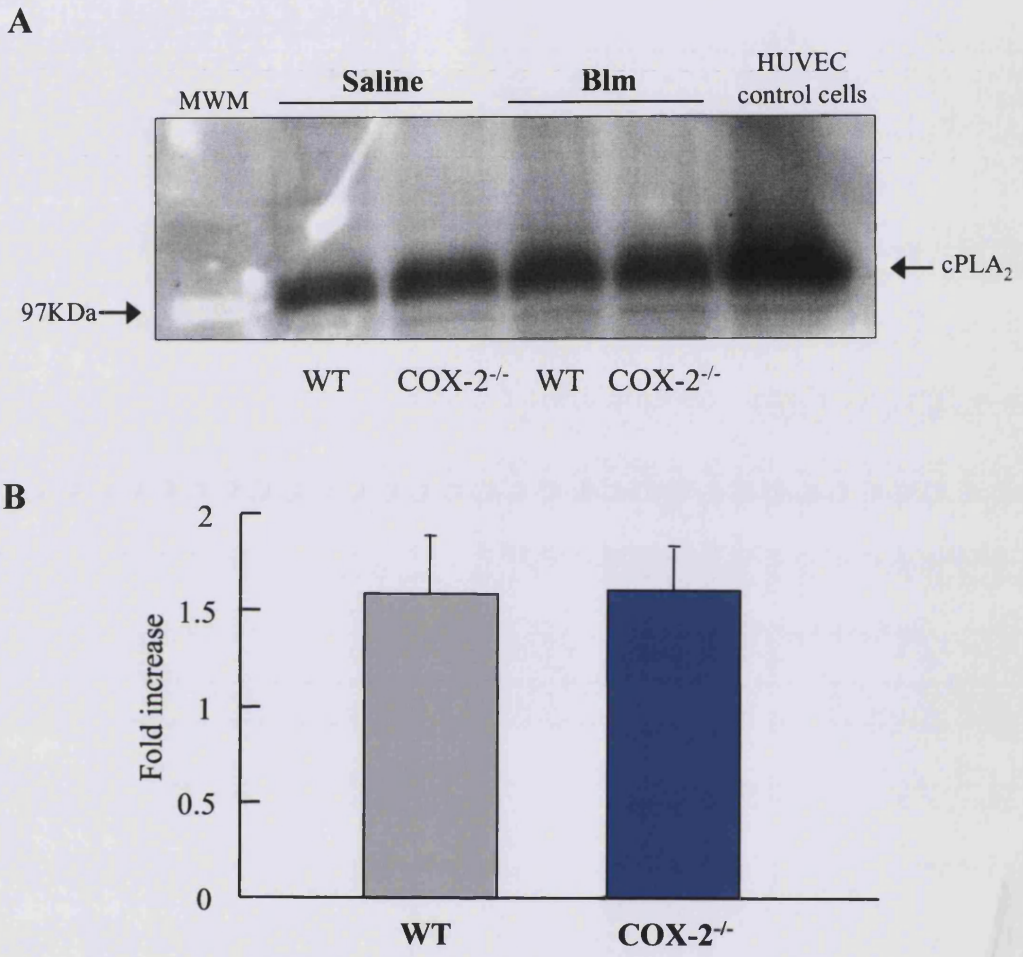


Figure 3.25. cPLA$_2$ expression in the lungs of wild type and COX-2$^{-/-}$ mice 14 days followings administration of saline or bleomycin
3.25A shows a representative western blot. Protein extracted from WT and COX-2$^{-/-}$ lungs following intratracheal instillation of saline or bleomycin was electrophoresed on SDS-PAGE gel and detected immunologically using a rabbit anti-mouse monoclonal cPLA$_2$ antibody. Band densities were quantified as described and **Figure 3.25B** shows the fold increase of cPLA$_2$ expression following bleomycin over saline control. Blots using 5-7 animals were used per group.

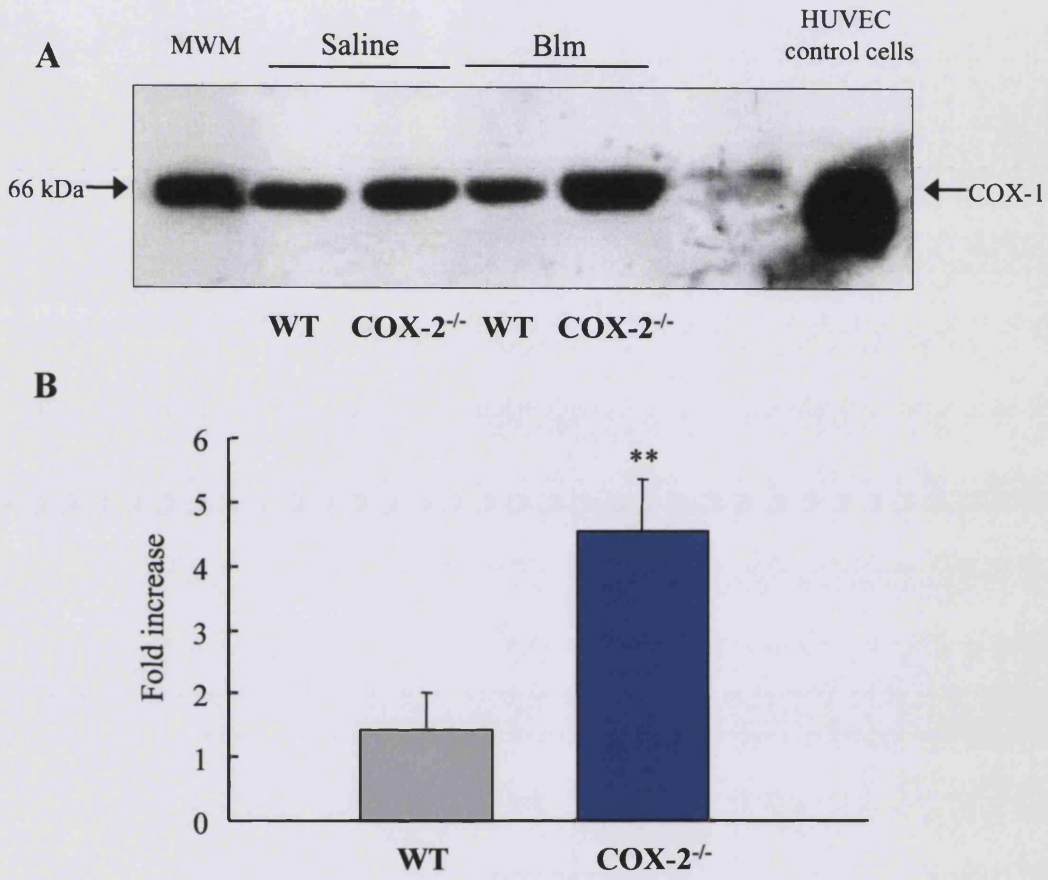


Figure 3.26. COX-1 expression in the lungs of wild type and COX-2$^{-/-}$ mice 14 days followings administration of saline or bleomycin

3.26A shows a representative western blot. Protein extracted from WT and COX-2$^{-/-}$ lungs following intratracheal instillation of saline or bleomycin was electrophoresed on SDS-PAGE gel and detected immunologically using a goat anti-rabbit polyclonal COX-1 antibody. Band densities were quantified as described and **Figure 3.26B** shows the fold increase of COX-1 expression following bleomycin over saline control. 5-8 animals were used per group ** indicates $p<0.01$ compared with WT PGE_2

3.20.1 PGE_2 production in COX-2 deficient fibroblasts

Briefly, pulmonary fibroblasts were isolated by explant culture of both WT and COX-$2^{-/-}$ lung tissue. Cells were grown to confluence in 12 well plates, quieced for 24 hours and then stimulated for 24 hours with TGF-β_1 (1ng/ml). After this time, the cell-conditioned media was removed and PGE_2 levels were measured as previously described.

Figure 3.27 shows that PGE_2 production in WT fibroblasts is significantly induced by the pro-inflammatory cytokine TGF-β_1 ($p<0.05$). Addition of the selective COX-2 inhibitor NS398 (5μg/ml) reduced basal PGE_2 production by 80% and the TGF-β_1-stimulated PGE_2 production by 90% ($p<0.001$ in both cases). This suggests PGE_2 production in these cells is mediated predominantly via COX-2. In comparison, COX-$2^{-/-}$ fibroblasts produce PGE_2 at or below the limit of detection (20pg/ml). This indicating a basal production of PGE_2 via 'housekeeping' levels of COX-1 only, which is not inducible by TGF-β_1, and is unchanged in the presence of NS398. Therefore fibroblasts do not appear to be a source of the increased BALF PGE_2 production described in these mice.

3.20.2 PGE_2 production in COX-2 deficient macrophage/monocytes

Due to the difficulty in isolating sufficient numbers of pulmonary macrophage/monocytes an established model routinely used in this laboratory was used to isolate peritoneal cells (Bellingan *et al.*, 2002). Briefly, mice were injected with the pro-inflammatory mediator thioglycollate (4%w/v) and after 5 days were killed and the peritoneum lavaged using PBS. Cells from both WT and COX-$2^{-/-}$ mice were isolated and seeded in 96 well plates, allowed to adhere overnight and then washed to remove red blood cells and non-adherent inflammatory cells. Fresh media was added, and after 24 hours the cell-conditioned media was removed and PGE_2 production assessed. Macrophages from four different mice per genotype were used in at least triplicate wells per experiment. The results are shown in **Figure 3.28**. PGE_2 production is measurable in both COX genotype macrophage/monocyte populations. However, levels of PGE_2 in thioglycolate-stimulated COX-$2^{-/-}$ cells are approximately 2.5-fold greater than that measured in stimulated WT cells.

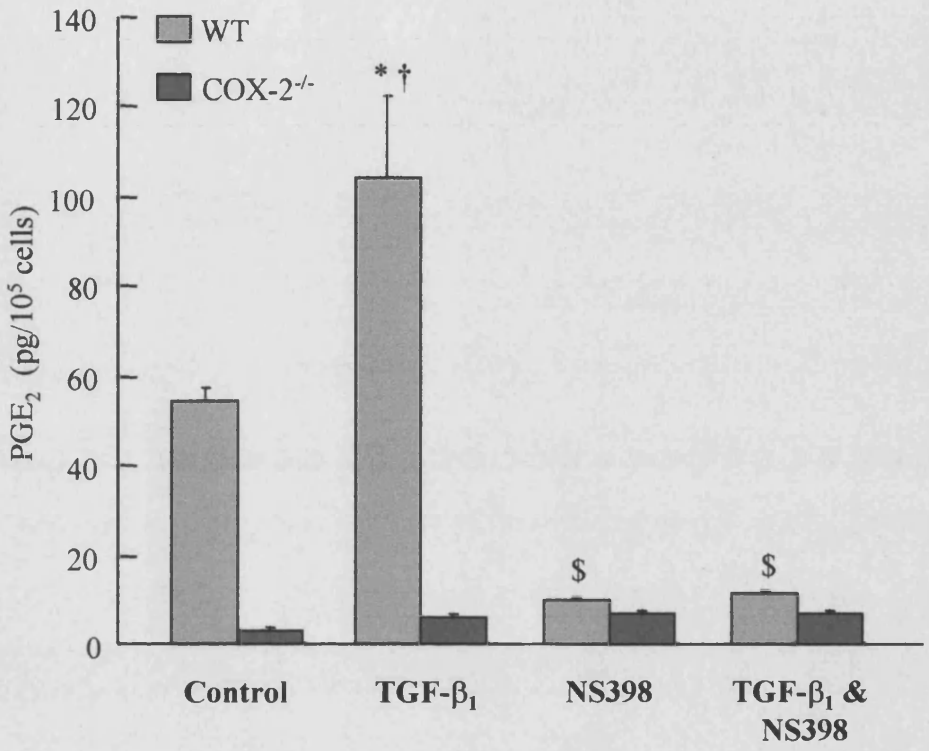


Figure 3.27. PGE_2 production in wild type and COX-$2^{-/-}$ fibroblasts
Fibroblasts were cultured with either 1ng/ml TGF-β_1 or the selective COX-2 inhibitor NS398 (5μg/ml), or both NS398 and TGF-β_1 for 24 hours. PGE_2 was measured in cell-conditioned media. Each bar represents the mean ±SEM for 6 replicate cultures. * indicates $p<0.05$ compared with WT control cells. † indicates $p<0.05$ compared with COX-$2^{-/-}$ TGF-β_1 treated cells. $ indicates $p<0.001$ compared with WT cells. Data is representative of 3 separate experiments.

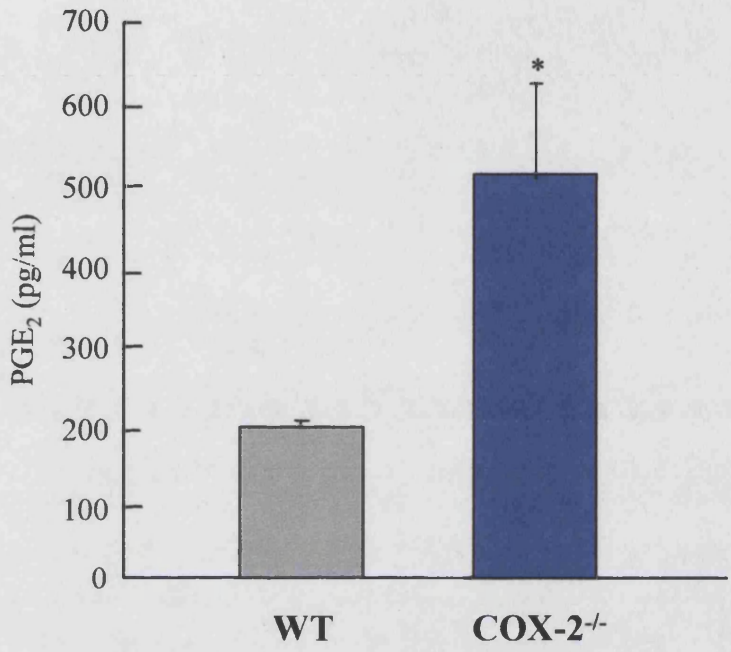


Figure 3.28. PGE_2 production in wild type and COX-$2^{-/-}$ macrophage/monocytes

Macrophages/monocytes were isolated 5 days after peritoneal injection with the inflammatory stimulant thioglycolate. Cells were cultured in serum-free conditions for 24 hours and PGE_2 measured in the cell-conditioned media. Each bar represents the mean ±SEM for a least 6 replicate wells of 4 separate animals per group. * indicates $p<0.05$ compared with WT control cells.

These data suggest that COX-$2^{-/-}$ macrophages/monocytes possess a compensatory mechanism, possibly increased production of COX-1, which results in an upregulation of PGE_2 production following stimulation.

3.21 Leukotriene C_4 expression in COX-2 deficient mice

Leukotrienes (LTs) are alternative metabolites of arachidonic acid, made not via the COX pathway, but via 5-lipoxygenase (5-LO). In the absence of COX-2, arachidonic acid metabolism may be shunted through the 5-LO pathway to produce increased levels of leukotrienes. These eicosanoids are known to be both pro-inflammatory and pro-fibrotic (Peters-Golden *et al*, 2002) and therefore it was important to measure their production in COX-2 deficient mice for two reasons: firstly, increased production of leukotrienes may contribute to the enhanced inflammatory response to bleomycin seen in COX-2 homozygous deficient mice, and secondly, if shunting could also occur in mice heterozygous for COX-2 the increased levels of pro-fibrotic leukotrienes may contribute to the enhanced fibrotic response to bleomycin observed in these mice.

LTC_4 was measured as a representative cysteinyl leukotriene which are the predominant class of LT expressed in mice (Moore *et al.*, 2000; Peters-Golden *et al*, 2002). Animals were instilled with either saline or bleomycin (1mg/kg) and after 7 days lungs were lavaged using PBS. After lipid extraction, BALF LTC_4 was measured using an EIA. **Figure 3.29** shows that BALF LTC_4 was detectable at baseline following instillation of saline alone in all three genotypes. BALF LTC_4 was increased in COX-$2^{-/-}$ mice compared with WT mice following instillation of saline alone, $p<0.05$. Following instillation of bleomycin, levels of BALF LTC_4 did not increase significantly in any genotype, and appeared to decrease in COX-$2^{-/-}$ mice although this difference was not significant. Together, these data suggests that shunting through the leukotriene pathway does not occur in either COX-$2^{-/-}$ or COX-$2^{+/-}$ mice, following bleomycin injury.

3.22 15-epi-Lipoxin A_4 expression following inhibition of COX-2 activity

It has been shown that COX-$2^{-/-}$ mice show an enhanced and persistent polymorphonuclear (PMN) alveolitis following instillation of bleomycin compared with both vehicle treated mice and those treated with the highly selective COX-2 inhibitor, NS398. A possible explanation could be that COX-2 has functions other than

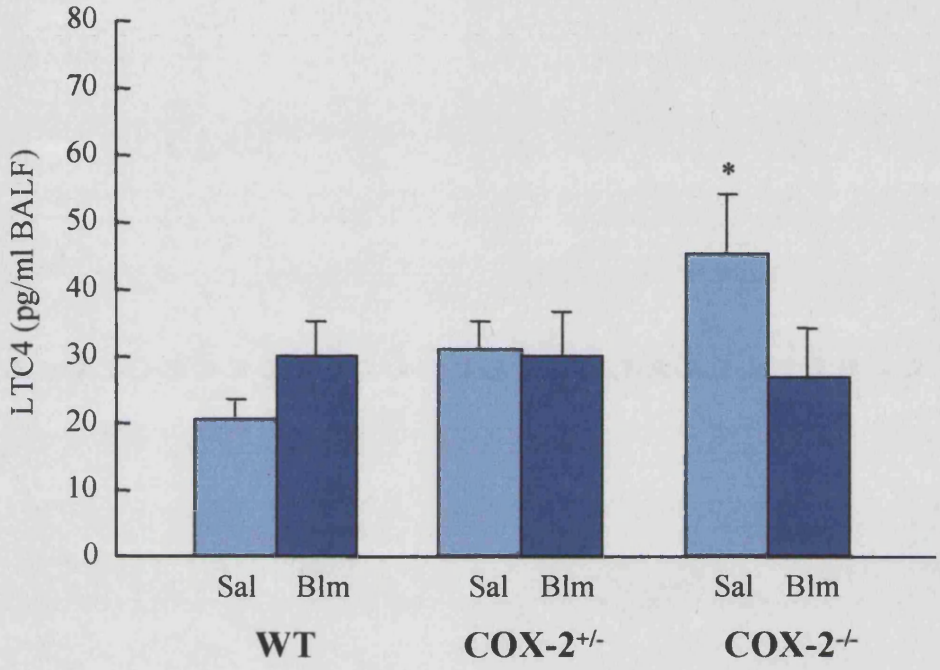


Figure 3.29. BALF LTC_4 levels 7 days following bleomycin-induced lung injury

Animals were lavaged with PBS 7 days following instillation of either saline or bleomycin (1mg/kg). BALF lipids were first extracted using C18 Sep-Pak light columns as described, and LTC_4 measured using a commercially available EIA (Cayman), according to the manufacturers instructions. Each value represents the mean ±SEM for 4-6 animals. * indicates $p<0.05$ compared with WT saline.

cyclooxygenase activity which are unaffected by NS398, but may aid in resolution of inflammation, and are absent in COX-$2^{-/-}$ mice

One potential candidate molecule is 15-epi-lipoxin A_4 (15-epi-LXA_4), which is a potent inhibitor of neutrophil binding, rolling and transmigration through the vascular endothelium. 15-epi-LXA_4 is generated from arachidonic acid by acetylated COX-2, via 15-*R*-HETE and 5-LO. High concentrations of 15-epi-LXA_4 are generated following acetylation of COX-2 by aspirin, however, it has been proposed that COX-2 may also be acetylated by endogenous acetylases or via non-enzymatic pathways involving reactive oxygen species (Chiang et al 1998). In addition, 15-epi-LXA_4 can also be generated via a COX-2-independent pathway involving cytochrome P450, although the degree of involvement of the cytochrome P450 pathway in aspirin-independent production of 15-HETE appears to vary between cell types (Claria et al 1996; Titos et al 1999). NS398 has been shown to reduce but not block 15-*R*-HETE production (Serhan et al 2000). Furthermore, the extent to which COX-$2^{-/-}$ mice can generate 15-epi-LXA_4 is not known. Therefore to investigate the role of 15-epi-LXA_4 in regulating PMN infiltration in bleomycin exposed mice, BALF levels were measured from vehicle-treated, and NS398-treated WT mice and COX-$2^{-/-}$ mice.

Figure 3.30 shows that 15-epi-LXA_4 is present, and measurable in normal mouse lung. All three experimental groups produced similar levels of 15-epi-LXA_4 following administration of saline alone. BALF 15-epi-LXA_4 levels following saline alone increase slightly at day 14 compared with day 7 in all genotypes, although this difference was significant in COX-$2^{-/-}$ mice only, $p<0.05$.

Following bleomycin challenge production of BALF 15-epi-LXA_4 increased at both 7 and 14 days. Vehicle-treated WT animals significantly induced BALF 15-epi-LXA_4 levels at both 7 and 14 days, $p<0.001$, as did COX-$2^{-/-}$ mice, $p<0.001$ day 7, $p<0.05$ day 14. WT mice treated with the selective COX-2 inhibitor NS398 showed increased BALF 15-epi-LXA_4 production at day 7, $p<0.05$, although this increase was not statistically significant at day 14, $p=0.07$.

Together, these data suggest that production of 15-epi-LXA_4 is unaffected by inhibition of COX-2, either pharmacologically or by gene deletion. Indeed, BALF 15-epi-LXA_4 levels were significantly higher in bleomycin-treated COX-$2^{-/-}$ mice than either vehicle

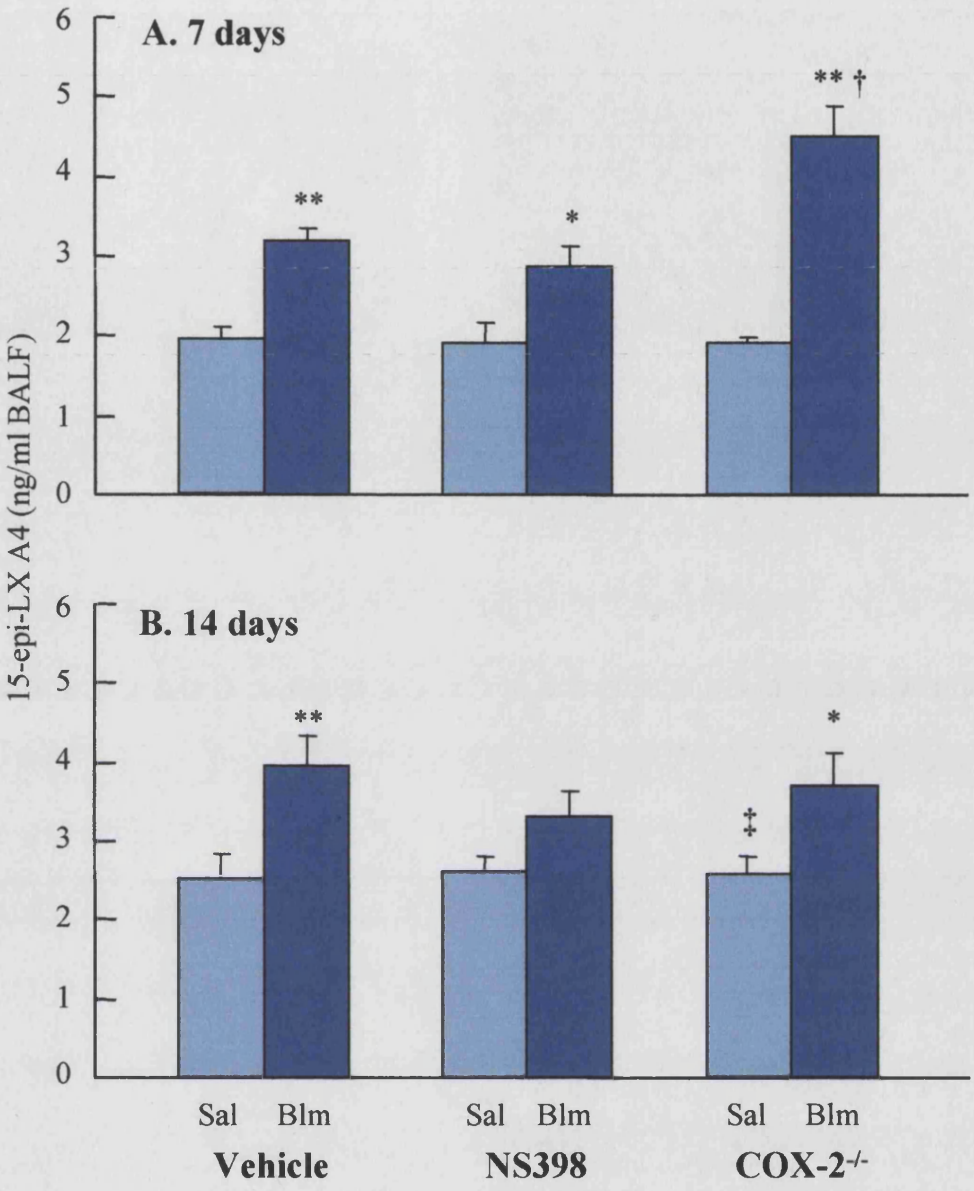


Figure 3.30. Effect of pharmacological inhibition of COX-2 and gene deletion on 15-epi-lipoxin A_4 levels in BALF 7 and 14 days following bleomycin injury

WT mice were treated twice daily with either the selective COX-2 inhibitor NS398 (3μg/ml) or vehicle. Both groups of wild type animals and COX-$2^{-/-}$ mice were instilled with either saline or bleomycin and after 7 and 14 days the lungs were lavaged using PBS. BALF lipids were extracted using C18 Sep-Pak light columns and 15-epi-LXA_4 measured using an EIA. Each value represents the mean ±SEM for at least 6 animals per group. * indicates $p<0.05$ and ** $p<0.001$ compared with instillation of saline alone, † indicates $p<0.05$ compared with both 7 day NS398 and vehicle blm, ‡ indicates $p<0.05$ compared with day 7 COX-$2^{-/-}$ saline.

or NS398-treated animals at day 7. These data suggest that a reduced capacity to synthesise 15-epi-LXA_4 does not mediate persistent and enhanced inflammation seen in COX-$2^{-/-}$ mice.

3.23 Summary: COX-2 homozygous deficient mice

Although this laboratory has previously shown COX-$2^{-/-}$ mice to exhibit an enhanced fibroproliferative response to bleomycin as assessed histologically (Keerthisingam, *et al* 2001), this is not mirrored in an increase in total lung collagen production (**Figures 3.17** and **3.18**). However, these animals do exhibit an enhanced inflammatory response to bleomycin characterised by an increase in PMNs and lymphocytes in the BALF (**Tables 3.2** and **3.3**). This section has shown that the enhanced inflammatory response is not due to shunting through the 5-LO pathway to produce pro-inflammatory leukotrienes, and nor is it due to a failure to produce anti-inflammatory 15-epi-LXA_4.

However, PGE_2 is known to be a pro-inflammatory mediator and COX-$2^{-/-}$ mice do show a compensatory upregulation in BALF PGE_2 synthesis following bleomycin injury (**Figure 3.16**). This compensatory upregulation of PGE_2 is apparent in macrophage/monocytes, possibly other cell types, but not fibroblasts derived from COX-$2^{-/-}$ mice (**Figures 3.27** and **3.28**). The increased expression of COX-1 in COX-$2^{-/-}$ whole lung tissue suggests that this may be the mechanism by which macrophage/monocytes are able to synthesise increased levels of BALF PGE_2 in COX-$2^{-/-}$ mice.

CHAPTER FOUR:

DISCUSSION

A growing body of evidence suggests that PGE_2 is an important negative regulator of fibroblast function in both the normal and fibrotic lung. It has been shown to be a potent inhibitor of fibroblast proliferation (Elias *et al.* ,1988; Bitterman *et al.*,1986), collagen synthesis (Goldstein and Polgar, 1982; Saltzman *et al.*, 1982), chemotaxis (Kohyama *et al.*, 2001), and more recently fibroblast-myofibroblast differentiation (Kolodsick *et al.*, 2003). Conversely PGE_2 has also been shown to promote degradation of newly synthesised collagen (Baum *et al.*, 1980; Brilla *et a.*, 1995).

PGE_2 is also a potent inhibitor of TGF-β_1-induced fibroblast proliferation and collagen synthesis (McAnulty *et al.*,1995; 1997) and despite increased levels of pro-fibrotic and pro-inflammatory mediators known to increase it's synthesis, levels of PGE_2 in BAL fluid from patients with pulmonary fibrosis have been shown to be 50% lower than in normal individuals (Borok *et al.*, 1991). Additionally, fibroblasts from the lungs of patients with pulmonary fibrosis show decreased synthesis of PGE_2 in the presence of TGF-β_1, TNF-α or IL-1 (Wilborn *et al.*, 1995; Keerthisingam *et al.*, 2001;Vancheri *et al.*, 2000; Marchand-Adam *et al.*, 2003). *In vitro*, this failure to induce synthesis of PGE_2 in response to an array of pro-fibrotic mediators has been shown to be associated with a decreased capacity to upregulate COX-2 (Wilborn *et al.*, 1995; Keerthisingam *et al.*, 2001), furthermore this leads to a more fibrogenic phenotype in fibrotic lung fibroblasts characterised by a loss in the anti-proliferative response to TGF-β_1 and an increase in procollagen production following stimulation with TGF-β_1 (Keerthisingam *et al.*,2001). Together these data provide indirect evidence to suggest that dysregulation of COX-2 expression and subsequent PGE_2 biosynthesis may play a key role in the pathogenesis of pulmonary fibrosis.

From this background, the overall aim of this thesis was to show a direct link between COX-2 and PGE_2 deficiency and the development of pulmonary fibrosis *in vivo*, using the well characterised bleomycin model of lung injury. This was done by inhibiting the function of COX-2, and therefore presumably PGE_2 synthesis, in two ways; pharmacologically using the highly selective COX-2 inhibitor NS398, and genetically using COX-2 deficient mice. In support of this hypothesis, a preliminary histological investigation of the lungs of homozygous COX-2 deficient mice from this laboratory showed an increased fibroproliferative response to bleomycin challenge compared with controls (Keerthisingam *et al.*, 2001).

4.1 Breeding strategy and mouse strain

The COX-2 deficient animals used in this study were generated on a C57Bl/6SV129 cross background (Dinchuk *et al.*, 1995). Female COX-$2^{-/-}$ mice are infertile due to a failure to ovulate and ovarian fibrosis (Morham *et al.*, 1995; Dinchuk *et al.*, 1995), however female heterozygotes remain fertile. The original breeding strategy of mating male COX-$2^{-/-}$ animals with female COX-$2^{+/-}$ mice aimed to maximise the numbers of COX-$2^{-/-}$ pups produced. However, this strategy produced no wild type littermates and therefore the COX-$2^{-/-}$ mice were provided with strain-matched C57Bl/6SV129 wild type animals (B197) by the supplier as an appropriate control.

As previously reported, litters from COX-$2^{-/-}$ animals were less frequent and smaller than wild type animals with less COX-$2^{-/-}$ mice available for use than predicted by Mendelian inheritance patterns (Morham *et al.*, 1995; Dinchuk *et al.*, 1995). COX-2 deficiency may have resulted in reduced conception as both COX-2 and PGE_2 have been shown to be important in sperm formation and sperm-oocyte fusion (Marshburn *et al.*, 1989; Joyce *et al.*, 1987). Furthermore, survival to the time of weaning and completion of genotyping was reduced in COX-$2^{-/-}$ animals, presumably due to renal failure as previously reported (Dinchuk *et al.*, 1995; Morham *et al.*, 1995).

To increase the numbers of available animals, heterozygous breeding pairs were used which additionally produced wild type littermate mice (B196). Both B197 and B196 mice represent valid wild type controls for studies using COX-2 deficient mice, and both strains have been used in previous publications (Keerthisingam *et al.*, 2001; Zeldin *et al.*, 2001). However, it was important to assess any phenotypic variation between the strains in response to bleomycin injury. No significant difference was seen between the strains in either body weight, or change in body weight following instillation of bleomycin, and total lung collagen production did not alter between strains. Although BALF PGE_2 levels were significantly higher in B196 mice compared with B197, both basally and following bleomycin injury, the 6.5-7-fold increase was similar in both strains (see **Figures 3.3 and 3.4**). Despite these similarities, the results for the two strains of WT mice were kept separate in all experiments and not combined.

Both B197 and B196 (including COX-$2^{-/-}$ and COX-$2^{+/-}$) strains of mice are on a mixed C57Bl/6SV129 background. Due to the difficulties in breeding described, back-crossing

onto an inbred strain of mouse was not considered feasible. However, variable segregation of litters towards either a C57Bl/6 or SV129 parentage has the potential to increase phenotypic variability in response to bleomycin instillation. Mouse strain is known to affect the fibrotic response to bleomycin, for example, C57Bl/6 mice exhibit the characteristic fibrotic response to bleomycin whereas BALBc mice do not (Schrier *et al.*, 1983; Ortiz *et al.*, 1999). However, bleomycin has been shown to lead to a similar inflammatory response and collagen production in both C57Bl/6 and SV129J mice at 14 days (Ortiz *et al.*, 1999) and C57Bl/6SV129 have previously been shown to respond in a similar manner to pure SV129J mice (Munger *et al.*, 1999). Furthermore, global analysis of gene expression in C57Bl/6 and SV129 mice has demonstrated a 67% homology in their response to bleomycin with an 80% homology in inflammatory response genes and a 75% homology in matrix remodelling genes (Kaminski *et al.*, 2000). Therefore variable segregation should not be a significant source of error.

4.2 Effect of bleomycin on PGE_2 production

PGE_2 is the major eicosanoid product of fibroblasts in the lung (Levine and Alam, 1979; Korn et al., 1983; Shindo *et al.*, 1988; Brock *et al.*, 1999) and has previously been shown to be increased in lung homogenate (Giri and Witt, 1985) and serum (Chandler and Giri, 1983) following bleomycin injury. This thesis has shown that administration of bleomycin also causes an upregulation of PGE_2 in the BALF of WT mice (**Figures 3.3, 3.4 and 3.9**). This increase is time-dependent; levels are significantly increased compared with saline alone after 3 days and continue to increase at days 7 and 14. By day 28 levels have decreased from the maximum, but remain raised compared with administration of saline alone (**Figure 3.9**). The increased synthesis of BALF PGE_2 for at least 28 days differs from levels of PGE_2 measured in serum. Following bleomycin, PGE_2 levels in serum peak earlier at day 4, but have returned to baseline levels by day 21 (Chandler and Giri 1983). This difference may reflect the local tissue generation of PGE_2 by different cell populations. It is possible that cells of the acute inflammatory response are responsible for much of the early PGE_2 generation measured both in BALF and serum, whereas resident lung cells such as fibroblasts and epithelial cells synthesise the later PGE_2 measured locally in BALF which is not detectable systemically in serum. This upregulation of BALF PGE_2 synthesis for at least 28 days is also significantly longer than that reported in BALF following intratracheal instillation of the transition metal oxide V_2O_5 (Bonner *et al.*, 2002). In this study PGE_2 levels peaked at day 1 but returned to baseline by 3 days

following injury (see **Table 4.1**). This may reflect the severity of injury and indeed the authors of this paper comment on a less severe injury response in mice compared to previous studies in rats.

4.3 Role of COX-2 in BALF PGE_2 production

4.3.1 Effect of pharmacological inhibition of COX-2 using NS398 on BALF PGE_2 production

NS398 is one of the most selective COX-2 inhibitors (Futaki *et al.,* 1993; Futaki *et al.,*1994; Warner *et al.,* 1999) and has previously been shown to be highly selective for COX-2 and to significantly inhibit inflammation-induced PGE_2 production *in vivo* at doses as low as 1-1.5mg/kg (Masferrer *et al.,* 1994; Payá *et al.*, 1997; Futaki et al., 1997), without affecting constitutive PG synthesis in the stomach (Masferrer *et al.*, 1994). At 1 and 10mg/kg NS398 has previously been shown not to inhibit COX-1 activity in mouse granuloma tissue (Gilroy *et al.,* 1998), and at doses of up to 30mg/kg body weight it has been shown not to inhibit COX-1 activity either systemically or in the stomach of mice (Wallace *et al.*, 1998). Thus a dose of 3mg/kg administered orally twice daily was used to maximise effects whilst maintaining selectivity. Therefore, any inhibitory effects on PGE_2 production in the lung by NS398 at the dose of 3mg/kg body weight used in this study are likely to have been mediated via COX-2.

Twice daily administration of NS398 almost completely inhibited BALF PGE_2 for at least 14 days following bleomycin-induced lung injury (**Figure 3.9A & 3.9B).** These data, together with western analysis of COX-1 and COX-2 levels in the lung following administration of either saline or bleomycin (**Figure 3.7**), supports the suggestion that PGE_2 synthesis following bleomycin injury administration is predominately COX-2 derived.

However, at day 28, 3mg/kg NS398 was insufficient to significantly inhibit BALF PGE_2 synthesis (**Figure 3.9C**). This may be due to several reasons, for example the development of tolerance to the inhibitor due to increased metabolism of NS398 with time, or positive feedback following inhibition of PGE_2 as both PGE_2 and NS398 have been shown to induce COX-2 (Pilbeam *et al.,* 1993; Lu *et al.,* 1995). In light of the data in $COX\text{-}2^{-/-}$ mice (see **Figure 3.16, 3.26** and **section 4.3.2**), compensatory upregulation of the remaining COX isoform(s) following pharmacological inhibition may also be a

possible explanation. To date, there have been no studies to suggest that NS398 can increase COX-1 activity, however previous studies using NS398 have not examined prolonged administration in mice. This explanation does appear unlikely however as western analysis has shown no increase in COX-1 expression in lung tissue at day 28 following treatment with NS398 (data not shown, personal communication Caroline Wheeler-Jones). Any possible role for COX-3 in PGE_2 synthesis in the lung would require further investigation but tools do not currently exist commercially to specifically examine its expression. However, it appears unlikely to be a major contributing factor to PGE_2 levels since expression of COX-3 in the lung, and many other tissues, has been shown to be minimal when compared with that in the brain (Chandrasekharan *et al.*, 2002), although it is currently unknown if expression of COX-3 in the lung may alter with injury.

A recent publication may suggest an alternative explanation. Gao and colleagues describe an alternative non-enzymatic pathway for formation of bioactive PGD_2 and PGE_2 independent of cyclooxygenase, and therefore unaffected by administration of NS398 (Gao *et al.*, 2003). Prostaglandin-like compounds termed isoprostanes (IsoP) have previously been shown to form in humans from the free radical-catalysed peroxidation of AA independent of COX (Morrow *et al.*, 1990a). Analogous to PGs, IsoPs contain E/D-type prostane rings which are similar to COX-derived PGs (Morrow *et al.*, 1999). Whilst the structures of these compounds are similar, IsoPs are formed from endoperoxides in which the majority contain side chains *cis* to the prostane ring (O'Connor *et al.*, 1984; Morrow *et al.*, 1999; Morrow *et al.*, 1990b) whereas the PG intermediate PGH_2 formed by COX contains side chains in the *trans* configuration relative to the prostane ring. A major IsoP is 15-E_{2t}-IsoP (8-isoPGE$_2$), but unlike PGE_2 is unstable and epimerises to PGE_2 by undergoing reversible rearrangement of the side chains that are initially *cis*, to the more stable *trans* configuration.

Using gas chromatography (GC) and mass spectrometry (MS) Gao shows that compounds with a retention time and molecular weight identical to COX-derived PGD_2 and PGE_2, (enantiomers, termed *ent*-PGE_2/D_2) are formed both *in vitro* and *in vivo* via the IsoP pathway. At baseline, levels of *ent*-PGE_2 were low, however following oxidative injury with carbon tetrachloride (CCl_4) levels of both *ent*-PGD_2 and *ent*-PGE_2 increased significantly in rats and represent up to 15% of the PGD_2 present and 3% of PGE_2 present. Furthermore, Gao and colleagues showed that liver tissue obtained from 19 day old COX-deficient (COX-$1^{-/-}$/COX-$2^{-/-}$) mouse pups harvested *in utero* was capable of synthesising

ent-PGD_2 and *ent*-PGE_2, an observation which may also be significant to this thesis (see **section 4.3.2**). Two further observations are made in this publication which may be of relevance; firstly in an unpublished observation Gao states that unlike PGE_2, *ent*-PGE_2 is a poor substrate for 15-PGDH (see **section 1.13**) suggesting that the metabolism of *ent*-PGE_2 is significantly different from PGE_2. Secondly, the paper raises the issue that the degree of suppression of PG production by NSAIDs varies depending on the eicosanoid measured. For example, high dose of non-steroidals such as 1.5g of aspirin to normal human volunteers is associated with a 90% reduction in thromboxane formation, greater than 80% reduction in PGI_2 but a no greater than 60% decrease in PGE_2 (Bippi and Frolich 1990; Rane *et al.*, 1978; FitzGerald *et al.*, 1983). Reasons for this discrepancy are unknown but in light of the findings that *ent*-PGE_2 can be formed in a non-COX mechanism, Gao postulates that in certain settings PGE_2 could be produced from IsoP intermediates in the presence of NSAIDs.

Although in the Gao study *ent*-PGE_2 may represent only 3% of the total PGE_2 synthesised, these observations may impact on this thesis in several ways: (a) bleomycin is known to cause oxidative stress (Kappus, 1987) which may be analogous to CCl_4 injury; (b) the specific EIA used to measure BALF PGE_2 levels throughout this thesis would be unable to differentiate between PGE_2 and *ent*-PGE_2 and therefore levels of both compounds are included in all measurements; (c) it is predicted that generation of PGE_2 via IsoPs would not be inhibited by NS398 used in this thesis; (d) if indeed *ent*-PGE_2 breakdown by 15-PDGH is reduced, levels may accumulate over time. By day 28 following administration of bleomycin and treatment with NS398, PGE_2 production is no longer inhibited compared with vehicle alone and these levels may represent increased quantities of *ent*-PGE_2 at this time. However, to examine this hypothesis BALF samples throughout the 28 day timecourse would need to be analysed for *ent*-PGE_2 using GC/MS as described which is not feasible for this thesis.

4.3.2 COX-2$^{-/-}$ mice and BALF PGE_2 production

COX-$2^{-/-}$ mice are incapable of upregulating COX-2 protein following bleomycin injury (**Figure 3.14**), and it was therefore hypothesised that these mice would be unable to upregulate synthesis of PGE_2. However, it has been shown that following administration of bleomycin this is not the case. Basal levels of BALF PGE_2 following instillation of saline alone were low and did not differ between genotypes (**Figure 3.16**), however, contradictory to the hypothesis, at all timepoints studied BALF PGE_2 was significantly

increased in COX-$2^{-/-}$ mice compared with saline alone (**Figure 3.16**). Indeed, at 14 and 28 days following injury, PGE_2 was significantly increased compared with bleomycin–exposed wild type animals. This significant upregulation of PGE_2 synthesis seems initially contrary to previously reported studies in the lungs of COX-$2^{-/-}$ mice which are summarised in **Table 4.1**. Studies by both Bonner and Zeldin showed reduced expression of PGE_2 compared with wild type animals following exposure to V_2O_5 and lipopolysaccharide (LPS) respectively (Bonner *et al,*. 2002; Zeldin *et al.*, 2001), and Gavett *et al.*, reported unchanged levels of PGE_2 synthesis in COX-$2^{-/-}$ mice using a model of induced asthma following ovalbumin challenge, although there appeared to be a trend towards increased PGE_2 production following challenge in both WT and COX-$2^{-/-}$ mice (Gavett *et al.*, 1999). Additionally, in the colon, Morteau and colleagues showed that COX-2 deficiency prevents the increase of PGE_2 from tissue seen in wild type mice in a model of colitis induced by dextran sodium sulphate (DSS; Morteau *et al.*, 2000).

Stimulus used	Time following exposure	BALF PGE_2 WT mice (pg/ml)		BALF PGE_2 COX-$2^{-/-}$ mice (pg/ml)	
		Basal	Challenged	Basal	Challenged
LPS (Zeldin)	Immediately	N/A	128 ±10	N/A	58 ±8
Ovalbumin (Gavett)	24 hours	206 ±42	383 ±55	201 ±38	*300 ±84
V_2O_5 (Bonner)	24 hours	*500 ±125	*100 ±145	*200 ±25	*345 ±155
V_2O_5 (Bonner)	15 days	*460 ±105	*500 ±140	*290 ±25	*285 ±60
Bleomycin	14 days	233 ±65	1202 ±431	144 ±50	2975 ±518
Bleomycin	28 days	177 ±70	535 ±167	336 ±151	1489 ±163

Table 4.1 BALF PGE_2 levels following injury. *Approximate values only, exact figure not published. N/A not assessed

The data presented in this thesis have shown that instillation of a relatively low dose of bleomycin causes a more severe injury than either LPS, ovalbumin challenge or V_2O_5, indeed BALF PGE_2 levels in wild type mice 28 days following bleomycin are higher than that collected at early timepoints following LPS and ovalbumin challenge, and are comparable with PGE_2 levels 3-15 days following instillation of V_2O_5 (see **Table 4.1**). Therefore previous studies may not have reached an injury threshold whereby compensatory mechanisms are initiated in COX-$2^{-/-}$ mice. As shown in **Table 4.1**, Gavett and co-workers. showed a trend towards increased BALF PGE_2 synthesis in COX-$2^{-/-}$ mice 24 hours following the last ovalbumin challenge (Gavett *et al.*, 1999). Although this

50% increase does not reach statistical significance, these data indicate that the remaining COX isoform(s) is(are) capable of synthesising PGE_2. Ovalbumin challenge may not have a reached a threshold whereby compensatory mechanisms are able to synthesise significant amounts of PGE_2. This idea is supported by the observation that we saw no basal upregulation of PGE_2 following instillation of saline alone in COX-$2^{-/-}$ mice (**Figure 3.16**). Furthermore the compensatory response may be time dependent and 24 hours following the final ovalbumin challenge may not be sufficient to initiate compensatory mechanisms. This is supported by the observation that compared with WT animals, COX-$2^{-/-}$ mice show no increased synthesis of PGE_2 until day 7 following administration of bleomycin.

In addition to timing and severity of challenge, a third difference between this study and those discussed above (Gavett *et al.*, 1999; Morteau *et al.*, 2000 and Zeldin *et al.*, 2001; Bonner *et al.*, 2002) is the COX-$2^{-/-}$ animals used. The previous studies used COX-$2^{-/-}$ mice descended from those generated by disruption of exon 8 of the COX-2 gene (termed COX-$2^{ex8/ex8}$) by Morham and colleagues (Morham *et al.*, 1995). However, the mice used in this study are descended from those generated by Dinchuk and co-workers (Dinchuk, *et al.*, 1995) by the targeted disruption of exon 1 of the COX-2 gene (COX-$2^{ex1/ex1}$). Phenotypic variations between strains of knockout mice generated via disruption of different exons have previously been reported and may be of relevance in this study. For example, the phenotype of mice generated by the disruption of exon 8 of the TGF-β signalling protein Smad 3 (Smad$3^{ex8/ex8}$; Yang *et al.*, 1999) show quite distinct, and in part contradictory phenotypes compared with those generated by targeted disruption of exon 2 (Smad$3^{ex2/ex2}$; Zhu *et al.*, 1998). Smad$3^{ex2/ex2}$ mice developed metastatic colorectal cancer by the time they were 4-6 months old and Zhu and colleagues made no evaluation of immune function. In comparison, Smad$3^{ex8/ex8}$ knockouts were virtually tumour free but show impaired mucosal immunity and diminished T cell responsiveness to TGF-β. Although not previously reported, it is possible that this phenomenon may, in part, explain differences in response to bleomycin challenge seen in the COX-$2^{ex1/ex1}$ mice compared with other models using COX-$2^{ex8/ex8}$ deficient mice. Data examining body weight and change in weight following instillation of bleomycin further supports this suggestion. This thesis has shown that COX-2 genotype has no significant effect on either parameter, however the effect of COX-2 deficiency and body weight has been investigated on one previous occasion. Fain and colleagues reported that COX-$2^{+/-}$ mice (generated via disruption of exon 8) develop obesity compared to WT and COX-$2^{-/-}$ mice

(Fain *et al.*, 2001), however, this is not observed in the $COX^{+/-}$ mice used in this study, where COX-2 genotype had no effect on body weight or weight change following injury (**Figure 3.15**). However, whilst compensatory upregulation of PGE_2 synthesis has not been previously described in studies using $COX\text{-}2^{ex8/ex8}$ animals *in vivo*, fibroblasts derived from these mice have been shown to produced increased levels of PGE_2 both basally and following stimulation (Kirtikara *et al.*, 1998). This study will be described in greater detail in the next section.

4.3.2.1 Mechanism for upregulation of PGE_2 synthesis

Through the use of the selective COX-2 inhibitor NS398, it has been shown that the majority of BALF PGE_2 generated by wildtype mice following bleomycin challenge is COX-2 derived (**Figure 3.9**). However, it is apparent that $COX\text{-}2^{-/-}$ mice are capable of using a compensatory mechanism to generate significant levels of PGE_2 following injury in the absence of COX-2. The possibility of a further COX isoform or splice variant, in addition to the described COX-3, which would be inducible, but inhibited by selective COX-2 inhibitors has been discussed (Willoughby *et al.*, 2000), although there is no clear evidence to support its existence.

An alternative explanation may be the generation of *ent*-PGE_2 (see **section 4.3.1**) in $COX\text{-}2^{-/-}$ mice. However the levels of BALF PGE_2 synthesised by $COX\text{-}2^{-/-}$ mice at day 14 are 10-fold greater than the levels described at day 28 in B197 mice (**Figure 3.9** and **3.16**) and it appears unlikely that the 3% of total PGE_2 represented by *ent*-PGE_2 could account for the large increases seen in $COX\text{-}2^{-/-}$ compared with WT animals at this time. Furthermore, there is no reason to suggest that $COX\text{-}2^{-/-}$ mice would make increased levels of *ent*-PGE_2 compared with WT animals. Therefore, it is more likely that the compensatory upregulation of PGE_2 described in $COX\text{-}2^{-/-}$ mice originates from either increased substrate availability feeding through the COX-1 (and/or COX-3) pathway caused by increased levels of $cPLA_2$, the rate-limiting enzyme for the release of free arachidonic acid, or alternatively increased expression of COX-1/3 in the lungs of these mice. Again, COX-3-derived PGE_2 is unlikely to make a substantial contribution to overall levels in the lungs of $COX\text{-}2^{-/-}$ mice following bleomycin due to its low expression in the lung (Chandrasekharan *et al.*, 2002, see **section 4.3.1**), although it is currently unknown if COX-3 expression may be induced by injury.

Upregulation of PGE_2 synthesis in cells obtained from COX-2 deficient mice has previously been described. Kirtikara *et. al.* showed increased basal and IL-1β-stimulated release of PGE_2 in COX-$2^{ex8/ex8}$ deficient lung fibroblasts, with increased basal protein expression of both COX-1 and $cPLA_2$ in these cells. Following IL-1β treatment, COX-1 protein expression was not subsequently increased, whereas expression of $cPLA_2$ was further increased, mediating an additional rise in PGE_2 (Kirtikara *et al.*, 1998). From these data it was hypothesised that differences in COX-1, and specifically $cPLA_2$, expression may occur in the lungs of WT and COX-$2^{-/-}$ mice, both basally and following bleomycin-induced injury. But no differences were observed in basal expression of either $cPLA_2$ or COX-1 in the lungs of either WT or COX-$2^{-/-}$ mice following instillation of saline. Following bleomycin-treatment a 1.5-fold increase in $cPLA_2$ protein expression was observed, but did not differ between genotype (**Figure 3.25**). However, following injury the 4.5-fold increase in COX-1 protein expression in lung tissue from COX-$2^{-/-}$ mice was significantly greater than the 1.5 increase in WT animals (**Figure 3.26**). Therefore it is likely that in contrast to COX-$2^{ex8/ex8}$ deficient fibroblasts, the increased synthesis of PGE_2 seen in the lungs of COX-2 deficient mice following bleomycin injury was mediated, at least in part, via the increased expression of COX-1, and not by increased $cPLA_2$ activity. This observation raises further questions which this thesis has begun to address; firstly, what is(are) the cellular source(s) of increased COX-1-mediated PGE_2 production? And secondly, are increased source-cell numbers responsible for increased PGE_2 synthesis, or does COX-1 expression increase in these cells?

4.3.2.2 Cellular source of compensatory increase of BALF PGE_2

To investigate the mechanism of compensatory upregulation of PGE_2, populations of fibroblasts and macrophages were isolated. Both cell types are known to generate substantial amounts PGE_2 in the lung and are present in increased numbers in pulmonary fibrosis. Additionally, as previously discussed, COX-$2^{ex8/ex8}$ deficient fibroblasts have been shown to upregulate PGE_2 synthesis compared with WT and COX-1 deficient cells (Kirtikara *et al.*, 1998).

Basal levels of PGE_2 in wild type fibroblasts were significantly increased following stimulation with TGF-β_1 and reduced from control values by 80% in the presence of the selective COX-2 inhibitor NS398. Selective inhibition of COX-2 also reduced TGF-β_1-stimulated PGE_2 production by 90% (**Figure 3.27**). This suggests that WT cells

predominantly synthesise PGE_2 via COX-2, supporting the *in vivo* observations using NS398, and can be further induced by the addition of TGF-β_1. In comparison, COX-$2^{ex1/ex1}$ deficient fibroblasts in this study produced significantly less PGE_2 basally than wild type cells, showed no induction of PGE_2 synthesis following stimulation by TGF-β_1 and PGE_2 synthesis was unaffected by NS398. (**Figure 3.27**). This corresponds with data generated by Dinchuk and co workers who showed that COX-$2^{ex1/ex1}$ deficient primary embryonic fibroblasts produced over 10-fold less PGE_2 than wild type cells 8 hours following stimulation with serum (Dinchuk *et al.*, 1995). These differences in PGE_2 production seen in COX-$2^{ex1/ex1}$ fibroblasts further supports the suggestion that the two lineage s of COX-2 deficient mice are phenotypically different. However, these differences may also be due to the nature of cells involved; these experiments were conducted using primary cells, whereas the data described by Kirtikara and colleagues was obtained from transformed cells (Kirtikara *et al.*, 1998).

Similarly to COX-$2^{-/-}$ fibroblasts, a recent publication has shown that following stimulation with either LPS or IL-1, COX-2 deficient alveolar epithelial cells (AECs) are unable to upregulate PGE_2 synthesis compared with WT cells (Lama *et al.*, 2002). These data suggest that AECs are therefore unlikely to be a cellular source of compensatory upregulation of PGE_2 biosynthesis *in vivo*.

In comparison with fibroblasts and AECs, stimulated COX-$2^{-/-}$ peritoneal macrophages/monocytes showed significantly increased production of PGE_2 compared with wild type cells (**Figure 3.28**). Together these *in vitro* data suggest that the increased synthesis of PGE_2 seen in COX-$2^{-/-}$ mice is likely to be via macrophage/monocytes, but not fibroblasts or AECs, and the data in whole lung tissue suggests this is due to compensatory expression of COX-1 in these cells. This hypothesis is supported by two observations; (1) the increased synthesis of PGE_2 is unlikely to be generated by increased numbers of macrophages synthesising 'housekeeping' levels of PGE_2 via homeostatic levels of COX-1 in COX-$2^{-/-}$ mice as total macrophage number does not differ between the two genotypes; (2) a recent study has indeed described compensatory expression of COX-1 mRNA in peritoneal macrophages from COX-2 deficient mice (Zhang *et al.*, 2002). This study did not examine protein expression of COX-1 nor PGE_2 synthesis from these cells, nor COX-1 mRNA expression in COX-$2^{-/-}$ AECs or fibroblasts. However, it provides more compelling evidence that the increased synthesis of PGE_2 in COX-$2^{-/-}$ mice is COX-1

derived and generated by macrophages. This cell type-specific response may be due to regulation by different transcription factors in cell populations, or altered signalling pathways between cell types.

A criticism of this work may be that the macrophage/monocyte populations were obtained from mice challenged with thioglycolate, whereas the fibroblasts used were isolated from unchallenged animals. Thioglycolate challenge may have been sufficient to initiate compensatory upregulation of PGE_2 synthesis in macrophages/monocytes that had not occurred in fibroblasts whilst *in situ*. However, it is of note that stimulation with TGF-β_1 for 24 hours was not sufficient to induce COX-1-derived PGE_2 synthesis in COX-$2^{-/-}$ fibroblasts. Furthermore, PGE_2 production in other inflammatory cells types from COX-$2^{-/-}$ mice has not been examined, namely PMNs and lymphocytes, both of which are present in increased numbers in BALF from COX-$2^{-/-}$ mice compared with WT animals, and are known to produce PGE_2 (Morley *et al.*, 1979).

4.3.3 COX-$2^{+/-}$ mice and BALF PGE_2 production

By using mice heterozygous for COX-2, compensation between COX isoforms as observed in COX-$2^{-/-}$ mice appears to have been avoided. Primary embryonic fibroblasts cultured from COX-$2^{+/-}$ mice have previously been shown to produce intermediary levels of both COX-2 mRNA and PGE_2 compared with WT and COX-$2^{-/-}$ cells (Dinchuk *et al.*, 1995) In addition, this thesis has shown intermediate levels of COX-2 protein expression in COX-$2^{+/-}$ mice following bleomycin administration (see **Figure 3.14**). COX-$2^{+/-}$ mice show only a limited induction of BALF PGE_2 synthesis following bleomycin injury, which is significantly lower that WT mice at day 7. Levels peak at day 14 but by day 28 COX-$2^{+/-}$ mice produced only basal levels of PGE_2 with no significant increase following bleomycin compared with saline alone. In comparison, BALF PGE_2 remains significantly increased in both wild type and COX-$2^{-/-}$ animals. Interestingly, these observations are consistent with data in patients with pulmonary fibrosis where expression of COX-2 is reduced (Wilborn *et al.*, 1995; Keerthisingam *et al.*, 2000) and levels of BALF PGE_2 are 50% lower than non-fibrotic controls (Borok *et al.*, 1990). This suggests that mice heterozygous for COX-2 provide a good model of patients with pulmonary fibrosis in this respect.

4.4 Role of COX-2 and the inflammatory response to bleomycin

The role of COX-2 in the inflammatory response to bleomycin-induced lung injury is by no means clear-cut. Pharmacological inhibition of COX-2 using NS398 did not alter the inflammatory response to bleomycin in either BALF total cell numbers, or cell profiles. In contrast, genetic disruption of COX-2 in COX-$2^{-/-}$ mice resulted in an enhanced inflammatory response to bleomycin compared with WT, COX-$2^{+/-}$ and NS398-treated WT animals. By 28 days following injury PMN and lymphocyte numbers in WT, COX-$2^{+/-}$ and NS398-treated WT mice had decreased to baseline values as inflammation resolved (see **Table 3.2**). In contrast, BALF PMN and lymphocyte numbers were still elevated in COX-$2^{-/-}$ mice, and significantly increased compared with all other genotypes/treatment groups. By 84 days following injury, both BALF PMN and lymphocyte numbers in COX-$2^{-/-}$ mice remained elevated compared with saline controls, and were also significantly increased compared with WT mice (see **Table 3.3**). Furthermore, at this timepoint, numbers of BALF PMNs and lymphocytes appeared to be related to COX-2 deficiency as an intermediate response was seen in COX-$2^{+/-}$ mice. Although both BALF PMN and lymphocyte numbers were not increased following bleomycin injury in COX-$2^{+/-}$ mice, cell numbers were non-significantly increased compared with WT animals. This observation appears to be COX-2, but not PGE_2-dependent as BALF PGE_2 levels were similar in all three COX-2 genotypes at this time.

This persistent lymphocytosis and neutrophilia suggests that COX-2 is important in the development and/or resolution of inflammation. However, the inflammatory response of COX-$2^{-/-}$ mice to different models of injury has not been consistent. In addition to this study, several other groups have demonstrated evidence of enhanced inflammation in COX-$2^{-/-}$ mice: enhanced pulmonary eosinophilia and lymphocytosis in ovalbumin-induced asthma (Gavett *et al.*, 1999 and Carey et al., 2003); persistent chronic inflammation in a carrageenin-induced paw injury (Wallace *et al.*, 1998); enhanced colonic inflammation in DSS-induced bowel injury (Morteau *et al.*, 2000); and an increased inflammatory score in response to intratracheal instillation of V_2O_5 (Bonner *et al.*, 2002). However two studies have demonstrated no difference at early timepoints following injury (Zeldin *et al.*, 2001; Dinchuk *et al.*, 1995), and a further two studies have demonstrated reduced inflammation, firstly in collagen-induced arthritis (Myers *et al.*, 2000), and more recently in a sepsis model, COX-$2^{-/-}$ mice were shown to be resistant to endotoxemia induced by LPS instillation (Ejima *et al.*, 2003). This inconsistency in the

inflammatory response may relate to the varying insults administered and to the timepoint assessed, for example, the inflammatory response 1 hour following carrageenin-induced paw injury does not differ between COX-$2^{-/-}$ and WT mice, after 5hours COX-$2^{-/-}$ animals show a decreased inflammatory response (Dinchuk *et al.*, 1995; Wallace *et al.*, 1998) but after 1 week COX-$2^{-/-}$ mice show a persistent inflammation which had resolved in WT mice (Wallace *et al.*, 1998).

4.4.1 Mechanism for enhanced inflammation in COX-$2^{-/-}$ mice

The mechanism for increased and persistent inflammation following bleomycin instillation is therefore unclear. One potential explanation may be the abundance of pro-inflammatory leukotrienes via shunting of AA through the lipoxygenase (5-LO) pathway. In patients with aspirin-intolerant asthma shunting of AA through the 5-LO pathway has been considered a mechanism of increased inflammation following the inhibition of COX (Babu and Salvi, 2000). Therefore LTC_4 was measured as a representative cysteinyl leukotriene which are the predominant class of LT expressed in mice (Moore *et al.*, 2000; Peters-Golden *et al.*, 2002). But levels of BALF LTC_4 were low in COX-$2^{-/-}$ mice (**Figure 3.29**) and unaltered compared with WT animals. Therefore it is unlikely that increased production of pro-inflammatory LT are responsible for the enhanced inflammation observed. This is further supported by the observation that Gavett and colleagues showed no increase in LTB_4 production in COX-$2^{-/-}$ mice following ovalbumin challenge (Gavett *et al.*, 1999).

An alternative explanation may be the absence of anti-inflammatory eicosanoids due to COX-2 deletion. COX-2 has been shown to play an important role in the resolution of inflammation by generating anti-inflammatory cyclopentanone prostaglandins (15deoxy$\Delta^{12\text{-}14}PGJ_2$, Gilroy *et al.*, 1999) via PGD_2. Therefore a failure to synthesise 15-deoxy$\Delta^{12\text{-}14}PGJ_2$ may explain why COX-$2^{-/-}$ mice are unable to resolve their inflammatory response to bleomycin injury. However, NS398 has also been shown to inhibit 15-deoxy$\Delta^{12\text{-}14}PGJ_2$ synthesis and exacerbate inflammation (Gilroy *et al.*, 1999). Although this thesis has shown administration of NS398 unable to inhibit bleomycin-induced PGE_2 production by 28 days, and therefore presumably production of 15-deoxy$\Delta^{12\text{-}14}PGJ_2$, treatment with NS398 did not exacerbate the inflammatory response at either 14 or 28 days, and therefore it appears unlikely that differential synthesis of 15-deoxy$\Delta^{12\text{-}14}PGJ_2$ can explain the persistent inflammation seen in COX-$2^{-/-}$ mice.

It is possible that COX-2 has actions unrelated to the cyclooxygenase activity of the COX-2 molecule which are unaffected by NS398 but may aid in resolution of inflammation, and are absent in COX-$2^{-/-}$ mice. A potential candidate molecule is 15-epi-LXA_4 (see **Section 3.21, Figure 3.30**). Briefly, 15-epi-LXA_4 is a potent inhibitor of neutrophil binding, rolling and transmigration through the vascular endothelium. It is predominantly generated in transcellular biosynthesis of AA by acetylated COX-2 via 15-*R*-HETE and 5-LO, however it has been proposed that COX-2 may also be acetylated by endogenous acetylases or via non-enzymatic pathways involving reactive oxygen species (Chiang *et al.,* 1998). NS398 has been shown not to block 15-*R*-HETE generation (Serhan *et al.,* 2000) and therefore 15-epi-LXA_4 was measured to investigate if an absence in its synthesis may explain the failure to resolve the bleomycin-induced neutrophilia observed in COX-$2^{-/-}$ mice. All three experimental groups produced similar levels of 15-epi-LXA_4 following administration of saline alone which suggests that basal production is not COX-2-mediated. Following bleomycin, production of 15-epi-LXA_4 was significantly induced but unaffected by inhibition of COX-2, either pharmacologically or by gene deletion. Again, this suggests a COX-2-independednt mechanism, potentially though cytochrome P450-mediated generation of 15-*R*-HETE, although this has previously been described to vary between cell types (Claria *et al.,* 1996; Titos *et al.,* 1999). At 7 days following injury 15-epi-LXA_4 levels are significantly greater in COX-$2^{-/-}$ mice compared with both vehicle and NS398-treated animals. This may be explained by increased numbers of inflammatory cells present in COX-$2^{-/-}$ mice leading to increased transcellular synthesis of 15-epi-LXA_4. Therefore a reduced capacity to synthesis 15-epi-LXA_4 does not mediate persistent and enhanced inflammation in COX-$2^{-/-}$ mice.

However, the most likely explanation for the increased inflammation observed in COX-$2^{-/-}$ mice, at least at day 28, is as a result of the high levels of BALF PGE_2 which is a known neutrophil chemoattractant. Therefore the enhanced and persistent inflammation may not be a direct consequence of an absence of COX-2 either promoting or limiting resolution of inflammation, but the compensatory expression of COX-1 resulting in high levels of pro-inflammatory PGE_2 which is still significantly increased 28 days following instillation of bleomycin. However, previous studies using COX-$2^{-/-}$ mice have failed to demonstrate a consistent link between the severity of inflammation and prostaglandin levels following injury (**section 4.4** Gavett *et al.,* 1999; Wallace *et al.,* 1998; Morteau *et al.,* 2000; Bonner *et al.,* 2002; Zeldin *et al.,* 2001; Dinchuk *et al.,* 1995; Myers *et al.,*

2000; Ejima *et al.,* 2003) and two of these studies both describe an increased lung inflammatory response following V_2O_5 and LPS instillation in the presence of low levels of PGE_2 (see **Table 4.1**, Bonner *et al.,* 2002; Zeldin *et al.,* 2000). This inconsistency was also evident at day 84 following instillation of bleomycin where BALF PMN and lymphocyte numbers in COX-$2^{-/-}$ mice remain elevated compared with WT animals, despite similar levels of BALF PGE_2. Studies at timepoints intermediate to days 28 and 84 may provide additional information regarding the timecourse of PGE_2 upregulation in the lungs of COX-$2^{-/-}$ mice and the link between high levels of BALF PGE_2 and enhanced inflammation, however it appears as though COX-2 may have, as yet undetermined, prostaglandin-independent effects which contribute to the inflammatory response.

The data in this thesis also questions the association between inflammation and the development of fibrosis following bleomycin-induced lung injury. As described, COX-$2^{-/-}$ mice show an enhanced inflammatory response yet no increased production of lung collagen compared with WT animals. However, mice heterozygous for COX-2 show a comparable inflammatory response to WT mice yet develop a severe fibrotic response (see **Figure 3.18, Table 3.2** and **Section 4.5.3**). Numerous studies report an association between a reduced leukocyte recruitment into the lung and protection against the fibrotic effects of bleomycin compared with WT animals (Peters-Golden *et al.,* 2002, Howell *et al.,* 2001; Nagase *et al.,* 2002). However the converse is not true with COX-$2^{+/-}$ mice and may provide evidence to support recent suggestions that development of inflammation and fibrosis are not interdependent (see **Section 1.3.2**; Selman and Pardo, 2002; Gauldie, 2002a; Gauldie *et al.*, 2002b).

4.5 Role of COX-2 in development of pulmonary fibrosis

The role of COX-2 in the development of pulmonary fibrosis was assessed histologically at day 28 and 84 and biochemically by measuring total lung collagen at days 14, 28 and 84.

4.5.1 Effect of NS398 treatment on the development of pulmonary fibrosis

Both histological assessment and measurement of total lung collagen show that pharmacological inhibition of COX-2 using NS398 has no affect on the development of pulmonary fibrosis following instillation of bleomycin. At 14 days when administration of NS398 almost completely inhibits PGE_2 production (**Figure 3.9, section 4.3.1**), the

mean increase in lung collagen following bleomycin is not significantly increased in NS398-treated animals compared with vehicle-treated mice. In comparison, total lung collagen is significantly increased compared with saline alone in vehicle treated mice. By 28 days following injury, bleomycin causes a significant upregulation in total lung collagen in both vehicle and NS398-treated animals however there is no difference between treatment groups at this time.

Other studies using NSAIDs have shown that inhibition of COX ameliorated lung fibrosis following instillation of bleomycin (see **Section 1.6.2**, Thrall *et al.*, 1979; Mall *et al.*, 1991; Chandler and Young, 1989) and together, these results appears to contradict the hypothesis that PGE_2 is an anti-fibrotic mediator and its reduced expression can potentiate bleomycin-induced lung injury. However, the mechanism of either protection, or in this case, an unaltered response, may be due to the anti-inflammatory properties of COX inhibitors and the timescale over which they were administered. In all of the above studies NSAIDs were administered prior to instillation of bleomycin and throughout the timecourse, however when indomethacin was administered in only the fibrotic stage of the bleomycin model after day 10, an enhanced fibrotic response to bleomycin was observed (Moore *et al.*, 2000). This suggests that inhibition of COX during the initial phase of bleomycin injury could have reduced the proinflammatory effects of COX-2 which may have inhibited the development of fibrosis in these animals. In retrospect, it may have been better in this study to have delayed administration of NS398 to day 10.

4.5.2 COX-2$^{-/-}$ mice and the development of pulmonary fibrosis

In a previous histological study COX-2$^{-/-}$ mice have been shown to have an increased fibroproliferative response to bleomycin challenge compared with WT controls (Keerthisingam *et al.*, 2001). Using reverse-phase HPLC both wild type and COX-2$^{-/-}$ mice showed significantly increased total lung collagen typical of the model, but no difference was observed between genotypes, suggesting that ablation of COX-2 does not lead to enhanced collagen deposition (**Figure 3.17**). Whilst the biochemical data may seem to contradict the previous histological findings at day 14, it is of note that a fibroproliferative response to bleomycin was discussed which was a measure of both matrix protein staining and inflammation. Therefore the enhanced fibroproliferative response seen histologically may represent predominantly the enhanced inflammatory response at this timepoint (Keerthisingam *et al.*, 2001). The enhanced inflammatory

response in these animals may also explain the unaltered fibrotic response in COX-$2^{-/-}$ mice which is contrary to the original hypothesis. Increased numbers of neutrophils would result in increased generation of proteases and oxygen radicals promoting ECM degradation (Everts *et al.,* 1996) and studies using silica, coal dust or cigarette smoke have all demonstrated neutrophil-mediated tissue breakdown in the lung (Dhami *et al.,* 2000; Zay *et al.,* 1999). Increased numbers of lymphocytes could also affect fibroblast collagen production through the secretion of anti-fibrotic mediators such as INF-γ (Giri *et al.,* 1986). However, since PGE_2 is known to inhibit fibroblast collagen production, proliferation and fibroblast-myofibroblast transition, it is likely that high levels of BALF PGE_2 observed in COX-$2^{-/-}$ mice are primarily responsible for limiting the fibrotic response in these animals (**Figure 3.16, section 4.3.2**). Dietary induction of PGE_1 (the E series prostaglandin derived from dihomo-γ-linoleic acid) synthesis has been shown to limit bleomycin-induced lung collagen deposition (Ziboh *et al.,* 1997) and high levels of BALF PGE_2 may promote collagen degradation in COX-$2^{-/-}$ mice since PGE_1 has been shown to promote collagen degradation (Baum *et al.,* 1980). Therefore, increased levels of BALF PGE_2 may further alter the balance between collagen synthesis and breakdown in COX-$2^{-/-}$ mice.

By extending the timecourse to 84 days it was hoped that the compensatory upregulation of PGE_2 in COX-$2^{-/-}$ mice may have subsided, and therefore a more severe fibrotic response to bleomycin may have developed. BALF PGE_2 levels did decrease to basal levels by day 84 and total inflammatory cell numbers were unchanged compared with both WT and COX-$2^{+/-}$ mice. However, low PGE_2 was not sufficient to cause any further increase in the fibrotic response at this time; total lung collagen remained unchanged compared with levels seen at 28 days in COX-$2^{-/-}$ mice and were not different from WT controls. PMN numbers were no longer significantly increased in COX-$2^{-/-}$ mice at this time, however lymphocyte numbers remained elevated following bleomycin and may have remained a source of anti-fibrotic IFN-γ.

The inhibitory effects on other COX-2-derived prostanoids following either administration of NS398, or COX-2 gene deletion should also be considered. Decreased levels of other COX-2-derived prostanoids may play a role in the fibrotic and/or inflammatory response to bleomycin as intratracheal instillation of bleomycin has been shown to induce synthesis of 6-keto-$PGF_{1\alpha}$ (stable metabolite of PGI_2), thromboxane $(TX)B_2$ (stable metabolite of TXA_2) and $PGF_{2\alpha}$ in addition to PGE_2 in hamsters

(Chandler and Giri, 1983; Giri and Witt, 1985). TXA_2 has been shown to increase proliferation, and DNA and RNA synthesis in several cell types, including fibroblasts (Murota *et al.,* 1977; Mene *et al.,* 1990), and conversely, prostacyclin (PGI_2) decreases smooth muscle cell proliferation and collagen synthesis (Sinzinger et al., 1997; Gallagher et al., 1998). Many studies using COX-$2^{-/-}$ mice have not evaluated the effect of gene deletion on the formation of prostanoids other than PGE_2, however Bonner and colleagues measured levels of the pro-inflammatory PGD_2 following V_2O_5 challenge in both WT and COX-$2^{-/-}$ mice. Similar levels were reported in both genotypes suggesting that differences in PGD_2 production did not significantly contribute to the altered inflammatory and fibrotic responses reported in the lungs of COX-$2^{-/-}$ mice (Bonner *et al.,* 2002). A recent study has also examined the role of PGI_2 in ovalbumin-induced airway remodelling using IP gene-deficient mice. This study found that disruption of the IP gene enhanced the fibrogenic response to repeated allergen challenge by increasing levels of active TGF-β_1 in the BALF, and levels of hydroxyproline in the lung (Nagao et al., 2003). However, assuming that NS398 treatment and COX-2 gene deletion resulted in a deficiency in PGI_2 in this study, neither NS398-treated WT animals or COX-$2^{-/-}$ mice showed a similar enhanced fibrotic response to bleomycin.

In summary, the measurement of other COX-2-derived prostanoids following inhibition of COX-2 function, either genetically or pharmacologically, may be considered in the future, however PGE_2 remains the dominant prostanoid in the lung, and of lung fibroblasts (Levine and Alam, 1979; Korn et al., 1983; Shindon et al., 1988; Brock *et al.,* 1999).

4.5.3 COX-$2^{+/-}$ mice and the development of pulmonary fibrosis

With only limited induction of PGE_2, COX-$2^{+/-}$ mice demonstrate a more severe and more extensive fibrosis at day 28 than either wild type or COX-$2^{-/-}$, both histologically and biochemically (**Figures 3.20B,** and **3.18**). Although COX-$2^{+/-}$ mice show a trend towards increased collagen 14 days following bleomycin total lung collagen, this is not significantly increased compared with either wild type or COX-$2^{-/-}$ animals at this time indicating that this phenotypic response is time dependent and may be associated with peak levels of PGE_2 at day 14.

A criticism of the bleomycin model is that it is not a progressive model. It was hoped that the fibrotic phenotype observed in COX-$2^{+/-}$ mice may have lead to a progressive

model of fibrosis unseen in other mice. Animals were instilled with either saline or bleomycin and after 84 days the fibrotic response was assessed (also see **section 4.5.2**). However, the fibrotic response of COX-$2^{+/-}$ mice was maintained but not enhanced at 84 days, whilst a trend was seen towards increased collagen in the lungs of COX-$2^{+/-}$ mice compared with WT and COX-$2^{-/-}$ animals, it was no longer significant. Therefore COX-$2^{+/-}$ mice do not represent a progressive model of bleomycin-induced lung injury. This may have been due to the low dose of bleomycin used in this study, or the single injury. An alternative model may involve more than one instillation of bleomycin over the 3 month period which may better reflect patients with pulmonary fibrosis.

Despite the data at 84 days, data at day 28 demonstrates that a limited induction of PGE_2 in mice heterozygous for COX-2 results in an enhanced fibrotic response to bleomycin-induced lung injury, and represents a good model of patients with pulmonary fibrosis in these respects.

4.5.3.1 Mechanism of enhanced response in COX-$2^{+/-}$ mice

As previously mentioned (**section 4.4.1**) COX-$2^{+/-}$ mice show a 'normal' inflammatory response to bleomycin injury yet develop a more severe fibrotic response (**section 4.5.3**). Therefore it is unlikely that the inflammatory response itself can have triggered the extraordinary fibrotic response. PGE_2 is an anti-fibrotic molecule known to inhibit fibroblast proliferation, collagen production and differentiation and these data suggests that in mice heterozygous for COX-2, limited induction of PGE_2 is an important mechanism in the progression of fibrotic disease. Indeed, a reduction in levels of PGE_2 has previously been linked to a pro-fibrotic phenotype. Moore and colleagues reported that mice deficient in granulocyte-macrophage colony-stimulating factor (GM-CSF) show enhanced fibrosis following bleomycin which correlates with reduced levels of PGE_2 in both alveolar macrophages and whole lung homogenates from these animals (Moore *et al.*, 2000). In addition, as previously described (**section 4.5.1**) this study showed that administration of the COX inhibitor indomethacin to wild type animals 10-21 days following bleomycin enhanced the fibrotic response (Moore *et al.*, 2000). Furthermore, $5LO^{-/-}$ mice deficient in LT biosynthesis are protected from bleomycin–induced lung fibrosis (Peters-Golden *et al.*, 2002). One mechanism of protection is by increased synthesis of BALF PGE_2 produced via 'shunting' through the COX pathway. With reduced levels of COX-2 in mice heterozygous for the gene it is possible that AA may be preferentially metabolised through the 5-LO pathway to produce pro-fibrotic

leukotrienes and this may, in part, account for their enhanced fibrotic response. However, this was found not to be the case in COX-$2^{+/-}$ mice, where LTC_4 levels are low (see **Figure 3.29**) indicating no shunting of AA metabolism through the 5-LO pathway.

Although these data have shown PGE_2 deficiency to be an important mechanism in an enhanced response to bleomycin-induced fibrosis, it may not be the only mechanism involved in the increased collagen production in COX-$2^{+/-}$ mice. A complex interaction between both stimulatory and inhibitory mediators is known to contribute to the regulation of fibroblast proliferation and collagen synthesis. This is a tightly regulated process and a reduction in the levels of the inhibitory mediator PGE_2 seen in COX-$2^{+/-}$ mice may alter the balance towards stimulatory mediators such as TGF-β, TNF-α, IL-1, PDGF, IGF-1, or thrombin. It is possible that one or a combination of stimulatory mediators may be increased in the lungs of COX-$2^{+/-}$ mice which in turn may stimulate increased fibroblast proliferation and collagen production in the lung following bleomycin injury. Future work could be aimed at measuring pro-fibrotic mediators in BALF from these animals, for example TGF-β_1.

4.6 Summary and future work

The key findings of this thesis are:

The production of PGE_2 in BALF following administration of bleomycin is predominately COX-2-derived.

The reduced expression of COX-2 in COX-$2^{+/-}$ mice results in limited induction of PGE_2 following bleomycin induced lung injury and subsequently a more severe fibrotic response to injury than WT animals. In these respects COX-$2^{+/-}$ mice represent a good model of patients with pulmonary fibrosis where levels of COX-2 and PGE_2 have shown to be decreased, rather than absent in BALF and cells derived from the lungs of patients with pulmonary fibrosis (Borok *et al.*, 1991; Ozaki *et al.*, 1987; Wilborn *et al.*, 1995; Keerthisingam *et al.*, 2001; Vancheri *et al.*, 2000; 1: Marchand-Adam *et al.*, 2003).

Pharmacological inhibition of COX-2 function using the highly selective inhibitor NS398 inhibits BALF PGE_2 production for at least 14 days following bleomycin-induced lung injury. However by 28 days PGE_2 production is no longer significantly reduced and reasons for this are complex and currently unclear. At either 14 or 28 days following injury the fibrotic response is unaltered in mice treated with NS398 and this may reflect the dosing strategy for the drug. In retrospect, limiting the inhibition of COX-2 to the fibrotic phase alone, i.e. between days 10-28 after injury, may have been preferable.

The complete absence of COX-2 in COX-$2^{-/-}$ mice results in a severe and persistent inflammatory response to bleomycin injury characterised by an increase in BALF PMN and lymphocyte numbers. PGE_2 production is significantly upregulated in BALF macrophage/monocytes and this thesis suggests this is via compensatory synthesis of the remaining isoform COX-1. The enhanced inflammatory response may be a direct result of increased COX-1-derived PGE_2 production in the lung. COX-$2^{-/-}$ mice show an unaltered fibrotic response to bleomycin injury compared with WT mice and it is suggested that the enhanced inflammatory response and increased BALF PGE_2 levels limit the fibrotic response in these mice. Together with the data using COX-$2^{+/-}$ mice, this supports the suggestion that increasing or decreasing levels of PGE_2 in the lung modulates the fibrotic response to bleomycin as predicted; the decreased synthesis of PGE_2 in COX-$2^{+/-}$ mice resulted in an enhanced fibrotic response, whereas increased PGE_2 in COX-$2^{-/-}$ mice limited the fibrotic response in these animals.

Together these data have demonstrated not only the importance of using all available tools to determine the role of specific molecules in a disease model, but also the caution which should accompany the use of knockout mice where compensation must be an important consideration.

4.6.1 Future work

From these data future studies could:

Examine PGE_2 production in neutrophils and/or lymphocytes derived from COX-$2^{-/-}$ mice to investigate their contribution to compensatory upregulation of PGE_2 synthesis. Additionally, PGE_2 production in COX-$2^{-/-}$ fibroblasts derived from mice challenged with bleomycin could be examined to investigate if compensatory upregulation of PGE_2

synthesis was dependent on challenge *in situ*. Following this, COX-1 protein levels should be examined in all COX-2 deficient cell types which show compensatory upregulation of PGE_2 synthesis. This would provide direct evidence to show that the increased COX-1 expression observed in COX-$2^{-/-}$ lung tissue was causal for the increased PGE_2 production in COX-$2^{-/-}$ cells *in vitro*.

Measure other prostanoids which may play a role in the development of bleomycin-induced lung injury which would be inhibited either following administration of NS398 or absent in COX-$2^{-/-}$ mice.

Use conditional COX-2 knockout mice. In both NS398-treated WT and COX-$2^{-/-}$ mice the functional absence of COX-2 was not sufficient to prevent upregulation of PGE_2 synthesis in the lung. Indeed, COX-$2^{-/-}$ mice showed increased PGE_2 synthesis which this thesis suggests to be via compensatory expression of COX-1. Therefore the use of conditional COX-$2^{-/-}$ mice may avoid the compensatory effects observed in COX-$2^{-/-}$ mice. If compensation was avoided, these animals could also be used to investigate if the enhanced inflammatory response observed in COX-$2^{-/-}$ mice was a direct result of the absence of the gene, or as suggested, purely a consequence of the compensatory upregulation of PGE_2 synthesis. Conditional COX-$2^{-/-}$ mice may also represent a better model than COX-$2^{+/-}$ mice to investigate the specific roles of COX-2 and PGE_2 in the development of lung injury and fibrosis.

4.6.2 Therapeutic implications

Data in this thesis using COX-$2^{+/-}$ mice has shown for the first time that reduced expression of COX-2 and therefore PGE_2 biosynthesis results in a more severe fibrotic response to bleomycin-induced pulmonary fibrosis.

Therefore augmenting levels of PGE_2 in patients with pulmonary fibrosis may represent a novel treatment for the disease although sustaining elevated levels in the lungs may be difficult due to the rapid degradation of PGE_2. However, Borok and colleagues increased levels of PGE_1 in the lower respiratory tract by aerosol administration but this was very short-lived and levels declined to basal levels by 3 hours following administration (Borok *et al.*, 1991).

In addition to the problem of instability, the systemic side effects make long term administration of PGE_2 impractical, however an alternative suggestion is to overexpress

COX-2 (and therefore upregulate PGE_2 synthesis) by pulmonary gene transfer. This could be done using the LID vector, a non viral gene delivery system, which has been shown to have high transfection efficiency and minimal toxicity *in vivo* (Jenkins *et al.*, 2000; Jenkins *et al.*, 2003). Preliminary data has shown that using the LID vector sufficient COX-2 gene can be delivered to the lungs of mice in order to generate large quantities of PGE_2 (Jenkins, 2002; Jenkins *et al.*, 2002) and therefore COX-2 gene therapy may represent a potential therapy for the treatment of pulmonary fibrosis in the future.

Alternatively, rather than upregulating COX-2, or PGE_2, a selective PGE_2 receptor agonist may represent a potential therapeutic target. **Section 1.15** describes how many of the cellular actions of PGE_2 in the lung are mediated via the EP2 and EP4 receptors. Until recently, selective EP2/4 receptors were not long lasting enough to be used *in vivo*, however a recent publication has described CP-533,536, a highly selective EP2 receptor which when directly injected into bone induced bone healing in rat and canine models of bone fractures (Paralkar *et al.*, 2003). Whilst direct injection into the lung is not a feasible option, future work by the pharmaceutical industry may identify a similar molecule which could be successfully used in the lung.

CHAPTER FIVE:

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